Review

Interdisciplinary Approach by Hyperthermia and Cancer Gene Therapy

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Abstract: Hyperthermic therapy has been applied in many advanced malignancies, but tumor regression occurs only in thermo-sensitive cancers. To elucidate ways to overcome thermo-resistance and to improve the therapeutic efficiency of treating thermo-resistant cancers, we devised a novel application of cancer gene therapy in conjunction with hyperthermia. This strategy allows for selective cancer gene therapy under control of the heat-inducible HSP promoter. Heat-inducible activity of HSP promoter was examined in FM3A breast cancer cell line and MKN45 gastric cancer cell line using a luciferase assay reporter gene system. HSP promoter activity increased markedly following heat shock, and this increase depended on temperature and duration of treatment. Based on these results, we designed a suicide gene therapy using the Herpes simplex virus thymidine kinase gene ligated to the heat-inducible HSP promoter (HSP-tk). In in vitro cytotoxic assays HSP-tk transduced cells following heat treatment became 50,000 times more sensitive than either non-transduced cells or HSP-tk transduced cells without heat treatment to ganciclovir (GCV). Immunohistochemical analysis revealed that Fas-mediated apoptosis was involved in the synergistic killing effect of combination therapy. Next, we examined the efficacy of HSP-tk gene therapy in vivo, cancer cell lines implants in subcutaneous or intraperitoneal models of balb/c nude mice were targeted using the HVJ-anionic-liposome method. Significant inhibition of tumor growth was observed in HSP-tk transduced tumors following hyperthermia as more than half of treated-mice showed complete tumor eradication. Prolonged survival was also observed in HSP-tk transducted mice with hyperthermia. In contrast, non-transduced mice treated with or without hyperthermia showed no prolongation of survival. Recently, another group reported an in vitro study that HSP promoter-mediated gene therapy is an effective treatment for prostate cancer studied. Taken together, these results demonstrate that the combined gene and hyperthermic therapy may be a feasible treatment that can target HSP-expressing carcinomas, even in advanced cases.

Key Words: gene therapy, breast cancer, hyperthermia, HSP promoter, apoptosis

Introduction

Hyperthermia is a promising treatment option for advanced cancer, as radiofrequency capacitative heating devices have been used successfully for localized heating of tumors without noxious effects on
adjacent normal tissues. However, highly malignant cancers are usually thermoresistant due to HSP70 production. More than 300 cancer gene therapy clinical trials have been carried out, but an efficient and selective gene therapy has not been developed yet. Thus, we reasoned that hyperthermia in combination with gene therapy, designed to target HSP70, might be useful in improving quality of life and prolonging survival times in cases of advanced cancers. In this review, we summarize a novel strategy of suicide gene therapy in conjunction with hyperthermia based on our studies of breast and gastric cancer cell lines and later describe a possible future application for human cancer gene therapy using a modified liposome vector system.

Approaches to cancer gene therapy

One of the first strategies proposed for the use of recombinant DNA constructs in cancer patients that employed systemic injection of a non-toxic drug precursor; tumor cells were transduced with a specific enzyme gene whose product would convert a precursor into a toxic metabolite in the cells. In the first protocol to be approved using this strategy, brain tumors were transfected with a retroviral vector expressing the Herpes simplex virus thymidine kinase (HSV-tk) gene. Gancyclovir (GCV) delivering systemically was metabolized to cytotoxic gancyclovir triphosphates by cells expressing HSV-tk. Studies in a rat glioma model showed that marked tumor regression occurred even if only a small fraction of tumor cells were transfected with the HSV-tk gene. This cytotoxic effect on untransfected cells has been termed the "bystander effect".

While extensive testings of retroviral vector based-therapies have not revealed major signs of toxicity, it has failed to demonstrate significant effectiveness in clinical trials of cancer patients. Whereas adenoviral vectors used for localized treatment have had limited success. However the severe side effects including hepatotoxicity and demyelination can result from adenoviral-mediating HSV-tk gene therapy when using a constitutively active CMV promoter. Thus, a potential advantage of using a regulatable promoter-based gene therapy is that it might restrict suicide gene expression to tumor cells.

Tumor targeting by gene therapy under the control of promoter

Tumor targeting under the control of promoter is classified as shown in Table I. Previous studies in cancer gene therapy have investigated various cell type-specific promoters including mucin-1, c-erbB-2

<table>
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<tr>
<th>Table I. Classification of Promoter-based Molecular Targeting for Cancer Gene Therapy</th>
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<tr>
<td><strong>1. Cell-type Specific Promoter</strong></td>
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<tr>
<td>Hepatoma→AFP, Colon or gastric Cancer→CEA, Lung cancer→Myc-Max RE</td>
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<tr>
<td>Breast Cancer c-erbB-2, Mucin-1, L-plastin</td>
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<td><strong>2. Inducible Promoter</strong></td>
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<tr>
<td>Radiation→Radio-inducible promoter (Egr-1, ITA, p21)</td>
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<tr>
<td>Hyperthermia→Heat-inducible promoter (HSP70)</td>
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<tr>
<td>Drug →Tet-on/off system</td>
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<td><strong>3. Tumor Physiology-Specific Promoter</strong></td>
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<tr>
<td>Hypoxic, acidic or glucose-deprivation stress→HSP70, Egr-1, grp78, HRE (hypoxia-responsive element)</td>
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<tr>
<td><strong>4. Other Promoter</strong></td>
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<td>Tumor suppressor gene mutation→p53 response elements</td>
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(Her2/neu), secretory leucoprotease inhibitors, the hexokinase type II gene in breast cancer cells, carcinogenic embryonal antigen (CEA) in gastric and colon cancer cells, and alpha-fetoprotein (AFP) in hepatocellular carcinoma cells. In addition to cell type-specific promoters, the other promoter groups listed are specifically up-regulated by microenvironmental factors in tumor tissue or by spatially restricted inducers such as heat treatment and X-ray irradiation. Solid tumors show relatively poor vascularization, resulting in starvation of essential nutrients and oxygen. The autonomic nerve-cooling system by the reflexibly increased local blood flow is also lacking in tumor tissue. Furthermore, the density of pericytes and vascular smooth muscle cells is relatively low in tumor vessels compared to vessels in normal tissues. Hyperthermia creates a rise in the local temperature of tumor tissue exceeding that of the surrounding normal tissue. In order to establish resistance to hyperthermia, tumor cells express HSP70 protein following heat shock. As shown in Fig. 1, HSP70 expression in mouse mammary FM3A carcinoma cells, was not detected under basal conditions and was markedly induced by heat shock. In contrast, HSP70 was expressed in vivo even in the absence of heat shock and its expression was further enhanced FM3A implanted tumors. The in vivo heat shock-independent HSP70 gene expression might be the result of various stress conditions such as hypoxia or acidosis. Cancer gene therapy using HSP promoter is therefore likely an inducible and tumor-specific.

Thermotolerance and HSP expression

The colony formation assay confirmed that FM3A cells were heat-resistant, compared to their temperature-sensitive mutant tsFT101. Pretreatment of cells by heating at 45°C resulted in significant increase in the number of colonies in soft agar. In contrast, tsFT101 cells were thermosensitive and showed minimal thermotolerance (Fig. 2). Under control conditions, heat shock proteins were not detected in cultures containing FM3A or tsFT101 cells. After heat shock, inducible HSP70 appeared as a 72-kDa band in FM3A cells, 6 h after treatment. In contrast, HSP70 was not detected in heat-treated tsFT101 cells.

Increased expression of HSP70 which results from c-myc oncogene overexpression or p53 tumor suppressor gene mutation/null mutation is a poor prognostic marker of breast cancer as an indicator for a high risk of disease recurrence. Consistent with their, HSP producing FM3A cells displayed highly malignant phenotypes in vivo such as rapid growth, invasion and metastases, whereas tsFT101 cells could not be implanted in nude mice (data not shown).
Cells were incubated with various concentrations of GCV for 5 days, followed by cell survival quantitation as previously described\(^1\). Data are concentration of GCV yielding 50% growth inhibition (IC50) and therapeutic index comparing untreated cells with heat-treated cells.

Table II. In vitro cytotoxicity of GCV in parental and transduced FM3A cells after and without heat shock.

<table>
<thead>
<tr>
<th>Condition</th>
<th>IC50 (mg/l)</th>
<th>Therapeutic index</th>
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<tr>
<td>FM3A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without heat shock</td>
<td>250</td>
<td></td>
</tr>
<tr>
<td>With heat shock</td>
<td>250</td>
<td>1</td>
</tr>
<tr>
<td>FM3A-HSP(_{ik})</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without heat shock</td>
<td>250</td>
<td></td>
</tr>
<tr>
<td>With heat shock</td>
<td>0.006</td>
<td>50000</td>
</tr>
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Induction of HSP70 promoter activity by heat shock.

To estimate the levels of transcriptional activation of the HSP promoter, we performed luciferase assays using the human HSP promoter ligated upstream of the luciferase gene. Relative luciferase activity in FM3A cells increased by approximately 100 to 400 fold between 3 to 12 h after heat shock (Fig. 3A). In contrast, there was no significant increase in promoter activity in tsFT101 cells.

To confirm that increased HSP promoter activity resulted in production of heat shock factors, we performed gel mobility shift assays. These assays showed significant induction of heat shock factors in FM3A, but not in tsFT101.

We next examined HSP promoter activation by heat treatment in gastric cancer cell lines, MKN45 (Fig. 3B), NUGC3, and colon cancer cell lines, DLD-1, LoVo, 100-2000 fold activation was observed in all cell lines.

In vitro gene therapy

FM3A cells were retrovirally transfected with the expression unit containing HSV-tk DNA ligated to the HSP promoter (HSP-tk). Non-transfected and transfected cells were subjected to heat shock and treatment with GCV in various combinations. GCV (0-100 μg/ml) had no killing effect in HSP-tk-transfected FM3A (FM3A-HSPtk) in the absence of heat. Further GCV had no effect in non-transfected FM3A cells with or without heat treatment. In contrast, cells expressing HSP-tk and treated with heat shock were about 50,000 times more sensitive to GCV than cells that were not treated by heat shock (Table II).

To determine the mechanism of enhanced killing by heat shock, we examined to detect the presence of apoptotic cells in similar treatment condition for FM3A-CMVtk cells that were retrovirally transfected with HSV-tk gene constitutively activated by CMV promoter. Only 2 ± 0.4% of control FM3A-CMVtk cells showed evidence of apoptosis, and heat shock alone did not stimulate apoptosis (3 ± 0.5%) as detected by Hoechst dye staining. In contrast, treatment with GCV increased the percentage of apoptotic cells to 12 ± 1.9%, while treatment with both GCV and heat shock increased the proportion of apoptotic cells to 32 ± 6.6%.

We have evaluated the in vitro usefulness of the HSPV-tk/GCV system to overcome the insufficient therapeutic efficacy of hyperthermia for the treatment of mammary carcinoma cells. Blackburn et al. also investigated the effect of HSP promoter-oriented double suicide gene therapy in a prostate cancer cell line in vitro using adenoviral vector. In that first report using HSP promoter, heat treatment efficiently induced HSV-tk and cytosine deaminase fusion protein, and significantly reduced the survival of PC-3 cells in the presence of both GCV and 5-fluorocytosine.

In vivo gene therapy

Based on the above in vitro results, we applied hyperthermia and suicide gene combination therapy in vivo. HSP-tk vector was transfected using the HVJ-liposome method in nude mice that had been subcutaneously implanted with FM3A cells. FM3A cells are potentially highly malignant in vivo and large tumors with muscle invasion and para-aortic lymph node metastases develop within 3 weeks after cell inoculation. One week after cell implantation, tumors had reached about 1 cm in diameter. At this time, gene therapy or hyperthermia or combination treatment was commenced. Hyperthermia was administrated thrice every other day using a water bath (43°C, 30 min). Gene transfer was performed 3 times (days 1, 4,
8) by intratumoral HVJ-liposome vector injection. Four groups were treated with GCV (25 mg/kg/day) for 2 weeks: In the control group (Group I), mice treated with hyperthermia (Group II) and mice treated with mock vector and hyperthermia (Group III), all tumors increased exponentially in size. In contrast, mice treated with the combination of HVJ-liposome-mediated HSP-tk gene and hyperthermia (Group IV) displayed a substantial suppression of tumor growth, and nine out of 10 mice showed a complete eradication of tumors 3 weeks after implantation (Fig. 4).

The survival rates of mice treated by various regimens are similar as above. Among mice challenged intraperitoneally with tumor cells (FM3A or MKN45), untreated mice or those treated with heat shock or HVJ-liposome-mediated mock vector died from the tumor burden within 1 to 2 weeks after first injection of the gene therapy vector. Treatment with HVJ-liposome-mediated HSP-tk without heat shock prolonged survival, however all such mice died by day 21. In contrast, mice treated with HVJ-liposome-mediated HSP-tk gene combined with heat shock survived substantially longer (80% of mice were disease-free at day 28).

Mechanisms of bystander effect

In addition to molecular targeting by appropriate promoter selection, enhancement of the bystander effect is also important for suicide gene therapy. This is because it is impossible for the in vivo gene delivery system to transfer the gene into all cells in relatively large tumors^25). Several successful bystander
effects have been reported to activate tumor immunity by cytotoxic T-cells using syngenic animal models. However, patients with advanced cancers are immuno-compromised hosts. Therefore, we examined the potential bystander effect of gene therapy with the GCV/HSV-tk system in combined with hyperthermia in a nude mouse model lacking T-cell immunity. Tumors from each treatment group (control, heat shock, gene therapy and combination therapy) were analyzed by hematoxylin-eosin staining. The number of apoptotic cells exhibiting shrunken nuclei and apoptotic bodies increased in the combination group, whereas there was no increase in apoptotic cells in control, hyperthermia or gene therapy group (Fig. 5). We confirmed these binding by analyzing TUNEL stained sections. Additionally, leukocyte infiltration was not observed in any groups. These results suggest that the synergistic tumor cell killing effect of combination therapy depends upon apoptosis, but not on NK cell or T-cell mediated tumor immunity.

To test which apoptotic signal pathway is involved in enhancement of bystander effect, we analyzed immunohistochemical staining for FasL and Fas which are representative apoptotic molecules. In cells transduced with CMV-HSVtk suicide gene (this promoter activity is not markedly influenced by heat shock), IC50 data showed that combination therapy is 15 times effective on the killing of FM3A cells without promoter activation. Immunohistochemically stained control tumor cells and tumor cells treated with heat shock alone were negative for FasL. However, treatment with gene or combination therapy induced FasL expression in a proportion of FM3A cells. In contrast, Fas expression was evident in most cells of tumors treated by heat shock or by combination therapy, but not in control or gene therapy group. To further-clarify the involvement of Fas signal pathway, anti-Fas (P2) antibody (1:200), which neutralizes Fas-FasL interaction, or non-immune IgG as a negative control, was added to the medium. IC50 in P2 treated cells increased from 0.006 to 0.2 μg/ml, suggesting that this blocking antibody protected cells against additional Fas-induced apoptosis in combination therapy.

Fig. 5. Histological presentation of tumor treatment effects by hematoxylin-eosin staining. Left, control; Center, heat shock; Right, combination therapy. Apoptotic cells are increased in combination therapy. Magnification, X400.
HVJ-liposome vector

Using the HVJ-liposome vector system, we demonstrated heat-inducible suicide gene therapy selectively affects mammary carcinoma and human gastric cancer xenografts in vivo. Adenovirus-mediated gene transfer also exhibits greater gene transfer rates in breast cancer and gastric cancer. However, aderoviral transfer may be accompanied by the development of immunity, and therefore its effect becomes restricted to regression of local tumors. The HVJ-liposome vector which was constructed from inactivated envelopes of the Sendai virus and liposome, has low immunogenicity and toxicity, thereby allowing repeated administrations. In this regard, Gaber et al. reported that hyperthermia enhances the rate of extravasation of liposomes from tumor vessels. Thus, the HVJ-liposome method has several advantages over other gene delivery systems for in situ transduction in cancer gene therapy.

Conclusion

Although both suicide gene therapy and hyperthermia are potentially effective, the combination strategy resulted in almost complete tumor regression. From a clinical point of view, human tumors are heterogeneous, composed of HSP70-expressing and non-expressing cells. Cancer cells lacking HSP70 expression are sensitive to hyperthermia, and those expressing HSP70 may be thermotolerant but have a robust response to gene therapy (Fig. 6). Thus, a very wide therapeutic range may be anticipated not only in vitro, but also in vivo, further suggesting that this combination regimen is a potentially suitable treatment modality for advanced cancer.

References


がん温熱遺伝子治療の開発にむけて

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要 旨：がん遺伝子治療は進行癌や難治性癌の治療において，副作用が少なくしかも効果的な治療となる可能性を秘めているが，現段階における治療成績は必ずしも満足いくものではない。一方，悪性腫瘍に対する温熱療法も，癌細胞における熱ショック蛋白（HSP）誘導に代表される温熱抵抗性機序によりその効果は現在では限られたものである。しかし癌自殺遺伝子治療と温熱療法を併用することにより，癌細胞に対するFas依存性アポトーシスを介した機序により，著明な治療効果増強が得られることが見出された。さらに遺伝子治療における副作用をなくす目的で，癌細胞の温熱耐性獲得時にHSP遺伝子のプロモーター活性が上昇することを応用した，腫瘍選択的な分子標的治療を新たに考案したので報告する。