Enhanced suicide gene therapy by chimeric tumor-specific promoter based on HSF1 transcriptional regulation

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Received 7 May 2003; accepted 13 May 2003

First published online 3 June 2003

Edited by Julio Celis

Abstract **Two tandem cassettes, one containing the telomerase reverse transcriptase gene (hTERT) promoter upstream of a constitutively activated form of heat shock transcription factor 1 (cHSF1) and followed by the other containing the heat shock protein 70B (hsp70B) promoter (HSE) upstream of the cytosine deaminase (CD) gene, could greatly enhance the efficiency of CD gene therapy while retaining tumor specificity in vitro and in vivo. This hTERT-cHSF1/HSE promoter could restrict gene expression in tumor cells and was about 1.5–3-fold more potent than the cytomegalovirus (CMV) promoter. hTERT-cHSF1/HSE-CD transfection led to tumor cells more sensitive to 5-fluorocytosine compared with hTERT-CD and its toxicity was comparable to that of CMV-CD. Besides enhancement of promoter activity, cHSF1 overexpression itself could enhance the bystander effect of CD gene therapy that could be reversed by anti-Fas antibody. This system also led to activation of stress-related genes such as hsp70 in tumor cells, which in the presence of cell killing by the cytotoxic gene is a highly immunostimulatory event. Furthermore, a more potent anti-tumor effect of hTERT-cHSF1/HSE-CD was observed in nude mice inoculated with Bcap37 cells. No obvious activity of the hTERT-cHSF1/HSE promoter was observed in normal tissues after intravenous administration. These results indicate that the hTERT-cHSF1/HSE promoter is highly tumor-specific and strong with potential application in targeted gene therapy, and therefore may be useful for construction of vectors for systemic therapy.**

Key words: Telomerase reverse transcriptase; Heat shock factor; Cytosine deaminase; Tumor-specific promoter; Heat shock protein 70; Fas

1. Introduction

Tumor targeting is crucial to achieve successful effects in suicide gene therapy because the expression of the therapeutic gene may cause toxicity in normal tissues with systemic administration. It can be achieved by targeting of the delivery vector or restriction of expression of the therapeutic gene at the transcriptional level. Early clinical trials have demonstrated that, even using replicating vectors, the efficiency of gene delivery to tumors is generally very low [1]. It has been shown that the inclusion of tissue-specific, inducible or even potentially tumor-specific transcriptional elements can be very effective at targeting gene expression to tumor cells of several different histological types [2].

Currently used tumor-specific promoters include the tyrosinase gene promoter in melanomas [3], the carcinoembryonic antigen promoter in colorectal and lung cancer cells [4], the MUC1 promoter in breast cancer [5], and the E2F promoter in cancers that carry a defective retinoblastoma gene [6]. Nevertheless, although several reports suggest that relatively tumor-specific transgene expression is possible using these promoters, they also reveal several limitations. First, most of these promoters are limited to specific tumor histologies and cannot be used universally in tumors of various origins. To overcome this limitation, the telomerase reverse transcriptase (hTERT) promoter has recently been used to restrict gene expression to various tumor cells, because approximately 90% of tumors have telomerase activity, whereas most normal cells do not express telomerase [7]. Second, most of these promoters are much weaker than commonly used viral promoters such as the cytomegalovirus (CMV) early promoter, and the simian virus 40 (SV40) early promoter [8]. Consequently, their use is hampered by the problem of low expression. Gene expression driven by the hTERT promoter is also much lower than that driven by the CMV or SV40 promoter [9,10] and needs further improvement.

Previous researchers have induced tumor-specific death by selective expression of the cytosine deaminase (CD) or the herpes simplex virus thymidine kinase genes in tumor cells. However, few reports have demonstrated sufficient anti-tumor effects, even when tumor cells were introduced with specific promoter–suicide chimera genes in vivo by adenovirus (Ad), the most efficient vector for introducing foreign genes into target cells [11,12]. It is conceivable that low activity of cell type-specific promoters is not enough to induce sufficient drug sensitivity in vivo and expression of the suicide gene at a higher level is necessary for complete remission of tumors [13]. Therefore, we devised a transcriptionally regulating promoter system derived from the hTERT promoter which has broad tumor specificity with relatively low activity, to enhance tumor-specific CD gene therapy. In this promoter system, the

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Abbreviations: HSF1, heat shock transcription factor 1; hTERT, telomerase reverse transcriptase; CD, cytosine deaminase; hsp, heat shock protein; 5-FC, 5-fluorocytosine; DMEM, Dulbecco's modified Eagle's medium; CMV, cytomegalovirus; SV40, simian virus 40; RT-PCR, reverse transcriptase polymerase chain reaction; Ad, adenovirus

0014-5793/03/$22.00
doi:10.1016/S0014-5793(03)00606-9

heat shock protein 70B (hsp70B) promoter (HSE) was used to initiate CD expression, following another tandem cassette containing a constitutively activated form of heat shock transcription factor 1 (cHSF1) [14] which could activate HSE in the absence of stress, under the control of the tumor-targeting hTERT promoter.

Here, we demonstrate that this promoter system significantly enhances tumor-specific suicide gene therapy in vitro and in vivo and may be useful for systemic therapy.

2. Materials and methods

2.1. Cell lines

Human hepatocarcinoma cell lines Hep3B and HepG2, human cervical cancer cell line HeLa, human breast cancer cell line MCF-7, human colorectal cancer cell lines SW620 and HCT-116BG, human normal amnion cells WISH, and human fetal lung fibroblasts HFL-1 were purchased from ATCC (American Tissue Culture Collection, Rockville, MD, USA). Human breast cancer cell line Bcap37 was purchased from Shanghai Cell Collection and grown in RPMI 1640 supplemented with 10% fetal calf serum (Gibco BRL, Grand Island, NY, USA), 4 mM glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin. Other cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL) supplemented with 10% heat-inactivated fetal calf serum.

2.2. Plasmids

The core hTERT promoter (−378/+78 bp) was a gift from Dr. Bingliang Fang (University of Texas M.D. Anderson Cancer Center, Houston, TX, USA) [9]. HSE and cHSF1 with a deletion between amino acid positions 202–316 of wild-type HSF1 are described in our previous work [14]. cHSF1 was cloned downstream of the hTERT promoter and followed by another cassette containing the indicated gene under the control of HSE. This promoter system is represented as hTERT-cHSF1/HSE. To assess promoter strengths, plasmids were constructed using standard techniques such that different promoters (CMV, hTERT, or hTERT-cHSF1/HSE) were placed upstream of either the luciferase gene in the pGL3-Basic vector (Promega, Madison, WI, USA) or the CD gene in the pcDNA3 plasmid (Invitrogen, Carlsbad, CA, USA).

2.3. Luciferase assay

Cells mentioned above were seeded in 24-well plates at a density of 10^5 cells/well. The next day, cells were transfected with 150 ng of luciferase reporter plasmid and 20 ng of pCMW-β-gal using Lipofectamine reagent (Life Technologies, Grand Island, NY, USA) according to the manufacturer's instructions. After transfection for 6 h, the mixture was removed, and the cells were incubated under standard conditions. Luciferase assays were performed 48 h later using a Luciferase Assay System Freezer Pack Kit (Promega) and a luminometer, the values being normalized in relation to protein concentration. Internal normalization of the transfection efficacy was performed using a Luminescent Detection Kit to detect β-galactosidase (BD Biosciences Clontech, Palo Alto, CA, USA).

2.4. Reverse transcriptase polymerase chain reaction (RT-PCR)

Total cellular RNA was isolated using TRIzol reagent (Life Technologies) according to the manufacturer's protocol. RT-PCR was performed with total RNA (2 μg) using the First Strand RT-PCR Kit (Stratagene, La Jolla, CA, USA). A cDNA equivalent of 1 ng RNA was amplified by PCR using primers specific for the target genes. The thermal cycles were: 94°C for 1 min, 50°C for 1 min, 72°C for 2.5 min for 30 cycles for CD (1.3 kb), 94°C for 1 min, 51°C for 1 min, 72°C for 3.5 min for 30 cycles for hsp70 (2.1 kb), 94°C for 1 min, 55°C for 1 min, 72°C for 2 min for 30 cycles for cHSF1 (1.2 kb). The primer sets used were as follows: CD, ATGTCGAATAACGCTTTACAAAC (forward) and TCAACGTTTGTAATCGATGGCT (reverse); hsp70, ATGTCGGTGGTGGGCATAGA (forward) and GGTCGTTCTTCAGATTCCTG (reverse); cHSF1, CGGAATTCCCTTGCTCGAGATGGATCTGCC (forward) and GGAAGATCTCTAGGAGACAGTGGGGTCC (reverse). The amplified products were fractionated on a 1% agarose gel containing 0.5 mg/ml ethidium bromide and DNA patterns were visualized under UV light.

2.5. In vitro cytotoxic assays

Cells were plated in 24-well plates at a density of 10^5 cells/well and transfected with 1 μg of the appropriate plasmid using Lipofectamine. Twenty-four hours after transfection, the culture medium was changed to fresh medium containing various concentrations of 5-fluorocytosine (5-FC; 0, 10, 50, 100, 500, 1000 and 5000 μM; Sigma, St. Louis, MO, USA). After 5 days of incubation, plates were counted for living cells using trypan blue exclusion cell counting to assess the levels of cell survival [15]. Cell viability in wells without 5-FC was considered 100%. The 50% growth inhibitory concentration (IC_{50}) of 5-FC was calculated using a curve-fitting parameter, and the results are represented as the means ± S.D. from three independent experiments.

2.6. Establishment of stable cell lines

Bcap37 cells were transfected with CMV-CD. G418 (100 μg/ml, Gibco) was added to the culture medium to select the transfected cells after 48 h incubation. The selection was continued for 14 days and the G418-resistant colonies were isolated and propagated under selection (100 μg/ml G418), which was designated Bcap37/CMV-CD.

2.7. Adenovirus generation, purification and titration

Recombinant Ad vector alone (Ad) or that containing cHSF1 (Ad-cHSF1) was respectively generated by cotransfection of low-passage 293 cells with shuttle plasmid pCA13 or pCA13-cHSF1 and adenoviral packaging vector pBHGE3 (Microbix Biosystems, Toronto, ON, Canada). Transfections were performed using Effectene™ (Qiagen) according to the manufacturer's protocol. Individual plaques produced from recombination events were isolated and validated for the presence of the cHSF1 gene and absence of the adenoviral E1 gene (wild-type) by PCR. Appropriately validated plaques were purified by two subsequent passages through 293 cells. The final viral stock isolated was again verified by PCR. Quantities of Ad or Ad-cHSF1 suitable for in vitro studies were produced by infecting 293 cells with validated viral stock as described above, purified by cesium chloride gradient ultracentrifugation, and dialysis. The preparation of Ad or Ad-cHSF1 was titrated by plaque assay using 293 cells.

2.8. Experiments in nude mice

Bcap37 cells (2×10^6) suspended in 100 μl of serum-free DMEM were inoculated subcutaneously and bilaterally into female BALB/c *nu/nu* nude mice 4–6 weeks old. After 10 days, when most of the tumors had reached 3–4 mm in diameter, the mice were randomly divided into four groups: control, hTERT-CD, CMV-CD and hTERT-cHSF1/HSE-CD, and intratumoral Lipofectamine-mediated transfer of these vectors was performed three times (days 1, 4, and 8). 5-FC (250 mg/kg) was simultaneously injected i.p. twice a day for 14 consecutive days. Every 4 days after the beginning of the injection, tumor sizes were measured for 5 consecutive weeks. The tumor volumes (mm^3) were calculated as length × $width^2/2$. All animal experiments were done with the approval of the Animal Research Committee at the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences.

2.9. In vivo gene expression assay

Plasmid DNA (pGL3-Basic, hTERT-CD, CMV-CD or hTERT-cHSF1/HSE-CD) was delivered into mouse organs by the hydrodynamics method [16]. Four to six BALB/c female mice were used in each group. Twenty micrograms of DNA in 1.6 ml saline was injected via the tail vein in a time range of 6–7 s. Eight hours later mice were killed and liver, lung, spleen, heart and kidney were extracted. One milliliter of lysis buffer (0.1 M Tris–HCl, 2 mM EDTA and 0.1% Triton X-100, pH 7.8) was added to the whole organ for kidney, spleen, lung and heart. For liver, the same volume of lysis buffer was added to a piece of liver with a wet weight of approximately 200 mg. Samples (0.3 ml) were centrifuged at 14 000 rpm for 10 min at 4°C and luciferase activity was measured in concentrated or diluted supernatants.

2.10. Statistics

Data are expressed as mean ± S.D. values. Student's *t*-test was applied to analyze the relationship between the different variables. Statistical significance was taken at $P < 0.05$.

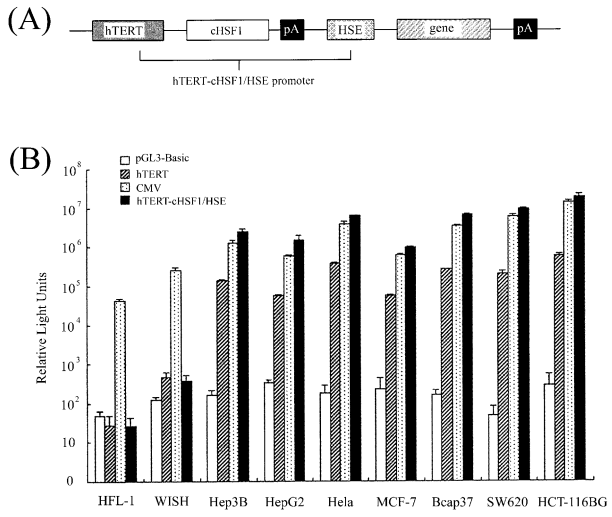

Fig. 1. In vitro promoter activity assay. A: The hTERT-cHSF1/HSE promoter sketch. PA, poly(A). B: Assessment of promoter activity in tumor or normal cell lines. Cells were transfected with luciferase reporter plasmid containing the luciferase gene under the control of different promoters (hTERT, CMV, hTERT-cHSF1/HSE). Luciferase assays were performed 48 h later. The values (mean ± S.D. for four assays) are represented as relative light units/mg of protein. The cells transfected by pGL3-Basic without enhancer/promoter were used as a negative control.

3. Results

3.1. In vitro comparison of promoter activity

cHSF1 could constitutively enhance HSE-mediated reporter gene expression in the absence of heat stress [14]. In this study, a 378-bp hTERT core promoter controlling cHSF1 expression was followed by a HSE-mediated expression cassette to construct the hTERT-cHSF1/HSE promoter system (Fig. 1A). The activity of different promoters (CMV, hTERT, hTERT-cHSF1/HSE) was assessed in seven cancer cell lines of various origins and two normal cell lines by the luciferase assay (Fig. 1B). The results show that hTERT promoter activity was significantly higher in tumor cells than in normal cells ($P < 0.01$) where its activity was not significantly different from that of pGL3-Basic. Despite its high tumor specificity, hTERT promoter activity was 10–30-fold less than CMV promoter activity in tumor cells. However, the hTERT-cHSF1/HSE promoter was about 1.5–3-fold more potent than the CMV promoter in tumor cell lines and remained very weakly active in normal cell lines. These results together demonstrate that the hTERT-cHSF1/HSE promoter is much stronger than the hTERT promoter in various tumor cell lines and could restrict gene expression specifically to tumor cells.

3.2. Enhanced tumor-specific suicide effect by the hTERT-cHSF1/HSE promoter in vitro

To test the efficacy of the hTERT-cHSF1/HSE promoter for selective tumor gene therapy, plasmids carrying the CD gene under the control of the CMV, hTERT or hTERT-cHSF1/HSE promoter were transfected into tumor cell lines Bcap37 or SW620 and normal cell lines HFL-1 or WISH. To confirm the operation of the hTERT-cHSF1/HSE promoter at the transcriptional level, RT-PCR was used to detect expression of the transgenes 48 h after transfection (Fig. 2). Strong mRNA for CD was detected in both tumor and normal cells after CMV-CD transfection, whereas it was very low or undetectable in normal cells after hTERT-CD or hTERT-cHSF1/HSE-CD transfection. Following the appearance of mRNA for cHSF1 in tumor cells transfected with hTERT-cHSF1/HSE-CD, a much higher level of mRNA for CD was detected than the hTERT-CD-transfected ones. hTERT-cHSF1/HSE-CD transfection also led to enhanced tumor-specific expression of hsp70, which was previously shown to have very potent immunostimulatory properties in anti-tumor vaccination settings.

These data confirm that the hTERT-cHSF1/HSE promoter enhances tumor-specific therapeutic gene expression at the transcriptional level and can induce endogenous heat shock gene expression. The latter induction will provide effective adjuvant functions for immunostimulation. It indicates that such a strategy would have an additional therapeutic advantage in vivo.

To analyze the 5-FC sensitivity of different promoters, the cells mentioned above were cultured in medium containing various concentrations of 5-FC for 5 days. The IC_{50} values are shown in Table 1. The results show that CMV-CD had significant toxicity in both tumor and normal cell lines. hTERT-CD only gave relatively low toxicity in Bcap37 and SW620 cells, but not in HFL-1 and WISH cells. hTERT-cHSF1/HSE-CD transfection specifically rendered tumor cell lines Bcap37 and SW620 about 23-fold more sensitive to 5-FC compared with hTERT-CD ($P < 0.0001$) and its toxicity was comparable to that of CMV-CD.

3.3. Effect of cHSF1 in the enhancement of the bystander effect in vitro

To test whether this enhancement of 5-FC sensitivity was

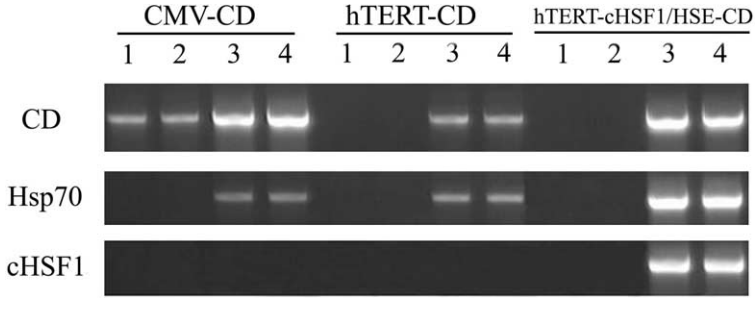

Fig. 2. The hTERT-cHSF1/HSE promoter is operative at the transcriptional level. Cell lines (normal cell lines: 1, HFL-1 and 2, WISH; tumor cell lines: 3, Bcap37 and 4, SW620) were transfected with plasmids containing the CD gene under the control of different promoters (CMV, hTERT, hTERT-cHSF1/HSE). Cells were harvested 48 h later and the mRNA expression of the CD or cHSF1 transgenes and of endogenous hsp70 were examined by RT-PCR.

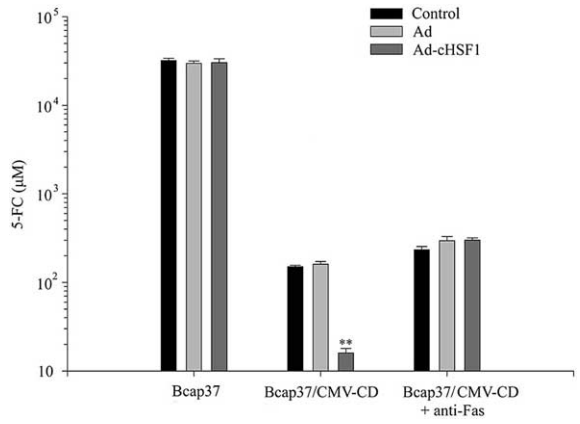

Fig. 3. Effect of cHSF1 in the enhancement of the bystander effect in vitro. Cells were plated in 24-well plates at a density of 10^5 cells/well and infected with Ad or Ad-cHSF1 at a multiplicity of infection of 50. Twenty-four hours later, cells were treated with various concentrations of 5-FC for 5 days in the presence or absence of anti-Fas antibody (1:200). Cell survival was assayed as described in Section 2. Significance is defined as $^{**}P < 0.01$ compared with Bcap37/CMV-CD cells infected with Ad. The values (mean ± S.D. for four assays) are represented as IC_{50} of 5-FC (μM).

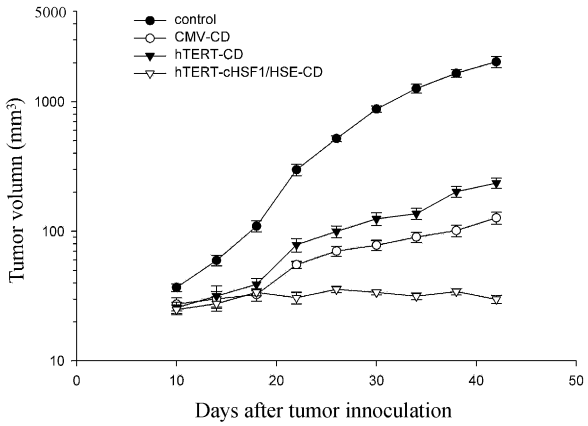

Fig. 4. In vivo therapeutic effect of CD gene driven by different promoters. Ten days after inoculation when the tumor diameters had reached 3–4 mm, 5-FC (250 mg/kg) was injected i.p. twice a day for 14 consecutive days. Tumor volume was measured every 4 days for 5 weeks and calculated as length × width²/2. Each data point represents the mean ± S.D. of eight samples. Significant suppression of tumor growth was observed after treatment with 5-FC in the hTERT-cHSF1/HSE-CD group compared with the CMV-CD group ($P < 0.05$, $n = 8$).

dependent on promoter activation only or cHSF1 itself also had some effect, we examined the effect of cHSF1 overexpression on non-heat-inducible CMV promoter. To obtain CD gene expression in all cells tested, the stable cell line Bcap37/CMV-CD was established and infected with Ad-cHSF1. The results show that Ad-cHSF1 infection decreased the IC_{50} of Bcap37/CMV-CD by 10 times (Fig. 3) while Ad infection had no such effect, so it was cHSF1 expression that made Bcap37/CMV-CD cells more sensitive to CD/5-FC gene therapy. It has been reported that cHSF1 overexpression could sensitize tumor cells to Fas killing [17]. Other reports have revealed that 5-fluorouracil (5-FU) induced apoptosis in vitro via the activation of the CD95/CD95L system [18]. To investigate whether the Fas apoptosis pathway was involved in the decrease of IC_{50} by cHSF1 overexpression, anti-Fas antibody which neutralized the Fas–FasL interaction, or non-immune IgG as a negative control, was added to the medium before treatment with 5-FC. IC_{50} values in anti-Fas antibody-treated Bcap37/CMV-CD cells returned from 16 ± 2 μM to 301 ± 18 μM, suggesting that this blocking antibody protected cells against additional Fas-induced apoptosis in the CMV-CD and Ad-cHSF1 combined treatment group. IC_{50} values in negative control cells were not changed by such treatment.

3.4. Enhancement of suicide gene therapy by the hTERT-cHSF1/HSE promoter in vivo

On the basis of the above-described in vitro studies, we applied this promoter system for suicide gene therapy in vivo. Different vectors (CMV-CD, hTERT-CD, or hTERT-cHSF1/HSE-CD) were transfected by the Lipofectamine reagent in nude mice subcutaneously implanted with Bcap37 cells. Fig. 4 shows growth curves of implanted tumors. After 5-FC treatment for 14 days, the control group without gene transfer showed a continuous increase in tumor size, while the other three groups all showed suppression of tumor growth. hTERT-cHSF1/HSE-CD led to a substantial suppression of tumor growth which was more effective than that of CMV-CD ($P < 0.05$).

3.5. Promoter activity assay in normal tissues

To test the specificity of the hTERT-cHSF1/HSE promoter in vivo, we studied the activity of different promoters in organs other than tumor tissue. Transgene expression using the hydrodynamics-based gene transfer method has been described in lung, spleen, heart, and kidney [16]. In this study, mice were injected via the tail vein with luciferase gene under the control of different promoters (CMV, hTERT, or hTERT-cHSF1/HSE). Eight hours later, the organs mentioned above

Table 1
5-FC sensitivity of cells transfected with plasmids containing the CD gene under the control of different promoters (hTERT, CMV, hTERT-cHSF1/HSE)

Cell line	IC_{50} of 5-FC (μM)[a]		
	CMV-CD	hTERT-CD	hTERT-cHSF1/HSE-CD
HFL-1	195 ± 5	22 387 ± 2 132	27 035 ± 3 017
WISH	118 ± 11	27 386 ± 3 321	24 130 ± 2 809
Bcap37	295 ± 24	3 427 ± 259	143 ± 36*
SW620	358 ± 47	4 135 ± 301	176 ± 16*

*$P < 0.0001$ compared with the IC_{50} of 5-FC in the same cell line transfected with hTERT-CD.
[a]In vitro cytotoxic assays were performed as described in Section 2. The results are represented as means ± S.D. from three independent experiments.

were extracted and analyzed for luciferase activity. Much lower levels of expression were observed with the hTERT and hTERT-cHSF1/HSE promoters than with the CMV promoter (Table 2) in all tissues, which were difficult to differentiate from the basal expression from the pGL3-Basic vector alone. The results indicate that the hTERT-cHSF1/HSE promoter had no effect on normal tissues in vivo and is suitable for systemic administration.

4. Discussion

A major challenge facing the routine use of gene therapy in the clinic is to achieve tumor-specific treatment. Tumor targeting can be accomplished at several levels, including direct injection into a target site, vector targeting, and tissue-specific gene expression [19]. Intratumoral injection is appropriate for the treatment of unresectable primary tumors, while systemic gene delivery may be necessary for the treatment of metastases. However, systemic delivery of therapeutic genes induces substantial toxicity to normal tissues, which suggests that either targeting vectors or targeting gene transcription may be essential for the systemic delivery of therapeutic genes in the clinic. Targeting vectors were developed based upon non-viral or viral gene delivery systems by various strategies [20], but even using replicating vector, the efficiency of gene delivery to tumors is currently very low [1]. Therefore, an efficient transcriptional targeting system may give some compensation.

Use of tumor-selective gene promoters is an attractive option for transcriptional targeting. There is now a growing list of tumor-specific promoters that are generally active only in the type of cancer from which they are derived or, at best, in a very limited number of tumor types. In contrast, the hTERT promoter should have the advantage of displaying activity over a broad range of cancers of very different etiology which augurs well for applications of hTERT promoter gene therapy in a wide range of human malignancies. In this study, we examined hTERT promoter-driven expression in cell lines from liver, breast, cervical, and colonic cancers. We showed that the hTERT promoter could drive gene expression specifically in tumor cells but its activity was 10–30-fold less than the CMV promoter. It is conceivable that expression of suicide genes at a higher level is necessary for complete rejection of tumors. To augment CD gene expression in tumor cells, we applied the HSF1/HSE responsive system. cHSF1 was placed downstream of the hTERT promoter. cHSF1 could transactivate the tandem HSE element in the absence of stress. Luciferase assay revealed that the hTERT-cHSF1/HSE promoter could produce stronger luciferase gene expression than the CMV promoter and its activity was strictly restricted to tumor cells. Cytotoxic assay demonstrated that the CD gene under the control of the hTERT-cHSF1/HSE promoter could selectively kill tumor cells 23-fold more efficiently than the hTERT promoter and was comparable to the CMV promoter.

HSE is the promoter of the hsp70B gene. Hsp70 is known to be abundantly expressed in human malignant tumors of various origins. It makes tumor cells resistant to apoptosis, which is especially deleterious because it not only enhances the spontaneous growth of tumors but also renders them resistant to host defense mechanisms as well as various forms of therapy [14]. Cancer gene therapy using the hsp70B promoter is considered to be tumor-specific, as well as inducible [21]. In our study, the hTERT-cHSF1/HSE promoter did not show any leaking in normal cells tested, because the HSE element in normal cells was not sufficient to initiate gene transcription. Our experiments clearly demonstrate that the hTERT-cHSF1/HSE promoter works at the transcriptional level and leads to tumor-specific cell killing comparable to that achieved with the CMV promoter alone.

In addition to the greatly enhanced promoter activity, the hTERT-cHSF1/HSE promoter could specifically express cHSF1 in tumor cells. cHSF1 expression could also transactivate endogenous HSE elements upstream of cellular stress response genes, such as hsp70. Previous studies have shown that expression of cHSF in tumor cells induces expression of hsp70 as well as other potentially immunostimulatory genes [22,23], which is very important for in vivo gene therapy. Induction of heat shock proteins is a highly potent immune adjuvant to tumor cell killing [24] and leads to the generation of long-term antitumor immunity.

To further study whether the enhancement of 5-FC sensitivity in vitro is dependent on promoter activity only, or cHSF1 itself may play a role, Ad-cHSF1 was used to infect Bcap37/CMV-CD cells, for the CMV promoter activity is not markedly influenced by heat shock [25]. IC_{50} data revealed that cHSF1 overexpression could decrease the IC_{50} of Bcap37/CMV-CD by 10 times. Fas-blocking antibody could block this effect. It has been demonstrated that the bystander effect of CD/5-FC gene therapy in vitro is mediated by 5-FU [26] which induces apoptosis via the activation of the CD95/CD95L system [18], so it may be the upregulation of Fas-mediated apoptosis events in Ad-cHSF-infected cells that was responsible for the decrease of IC_{50}. Other studies also found that Hsp70 overexpression could enhance Fas-mediated cell death [27] and cHSF1 overexpression could sensitize tumor cells to Fas killing [17]. Further studies about the interplay of Fas-mediated apoptosis with the (HSF1-activated) stress response are needed.

Furthermore, the therapeutic effect of the CD gene driven

Table 2
Activity of different promoters (hTERT, CMV, hTERT-cHSF1/HSE) in non-tumorous tissues

	Liver	Lung	Heart	Spleen	Kidney
pGL3-Basic	$3.31\times10^2\pm89.8$	$1.53\times10^2\pm26.8$	$2.79\times10^2\pm89.4$	$2.11\times10^2\pm68.7$	$3.04\times10^2\pm1.21\times10^2$
CMV	$8.69\times10^5\pm3.65\times10^4$	$6.21\times10^4\pm7.24\times10^3$	$4.28\times10^4\pm7.62\times10^3$	$7.12\times10^4\pm2.10\times10^4$	$2.56\times10^5\pm9.15\times10^4$
hTERT	$2.53\times10^2\pm52.8$	$1.06\times10^2\pm44.2$	$1.99\times10^2\pm69.7$	99.7 ± 15.6	$3.48\times10^2\pm90.3$
hTERT-cHSF1/HSE	$3.15\times10^2\pm86.7$	$2.97\times10^2\pm63.7$	$3.77\times10^2\pm1.06\times10^2$	$1.84\times10^2\pm39.5$	$3.11\times10^2\pm1.19\times10^2$

Luciferase reporter plasmid containing the luciferase gene under the control of different promoters (CMV, hTERT, hTERT-cHSF1/HSE) was delivered to mice by the hydrodynamic procedure. Liver, lung, heart, spleen, and kidney were harvested 8 h after DNA infusion and luciferase activity was determined in the organ extracts.
The values (mean ± S.D.) are represented as relative light units of luciferase per mg of total cell proteins. Mice transfected with pGL3-Basic without enhancer/promoter were used as a negative control. Much stronger luciferase activity was observed in each tissue tested after administration of the luciferase gene driven by the CMV promoter compared with the other promoters ($P<0.01$, $n=4$–6).

by the hTERT-cHSF1/HSE promoter was explored in nude mice. As expected, hTERT-cHSF1/HSE-CD led to substantial suppression of tumor growth and was more efficient than CMV-CD. The following reasons may account for the enhancement of CD/5-FC gene therapy efficiency by the hTERT-cHSF1/HSE promoter in vivo: (1) it allows increasing levels of CD to be expressed which will lead to very effective local tumor cell killing after 5-FC administration in vivo; (2) it leads to tumor-specific expression of cellular stress proteins, including hsp70 and natural killer cell receptors which could provide effective adjuvant functions for immunostimulation in vivo; and (3) it also enhances the bystander effect of CD/5-FC gene therapy by upregulating the Fas-mediated apoptosis pathway. Besides high efficiency for tumor gene therapy, the hTERT-cHSF1/HSE promoter also retained strict specificity for tumor tissues and was undetectable in normal tissues from various organs when administered intravenously. Thus, without the activation of the hTERT promoter in normal tissues, transactivation of the HSE element by innate HSF1 was not sufficient to initiate gene expression, so this promoter is promising in development of vectors for systemic administration.

In summary, we describe here a novel transcriptionally regulating promoter system which allows high level tumor-specific expression of potent cytotoxic genes combined with an inherent immunostimulatory capacity and an enhanced bystander effect for effective tumor-specific gene therapy.

Acknowledgements: This study was supported by the Key Project of the Chinese Academy of Sciences (No. KSCX2-3-06), the National Natural Science Foundation of China (No. 30120160823) and the State 863 High Technology R&D Project of China (No. 2001AA217031).

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