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β-Galactosidase as a marker of HSP70 promoter induction in Dunning R3327 prostate carcinoma cells

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Abstract Hyperthermia is known to improve the response of tumors to radiation or chemotherapeutic treatment when combined in multimodal strategies. The cellular response to hyperthermia is associated with the synthesis of heat shock proteins (HSP). To study the stress response in prostate cancer we have developed a clone of Dunning R3327 rat prostate carcinoma cells stably transfected with a gene construct containing the *E. coli* β-galactosidase gene driven by the *Drosophila* HSP70 promoter. The measurement of β-galactosidase serves as a rapid and semiquantitative assay of HSP70 gene activation. The Dunning cell clone showed evidence of incorporation of the HSP70/β-galactosidase construct within the genomic DNA by Southern blot analysis. When compared to mock-transfected control cells, the clone showed minimal baseline β-galactosidase activity, which significantly increased following a hyperthermic stress. The time course of β-galactosidase elevation following heat stress paralleled the time course of cellular HSP70 elevation by Western blot analysis. These stably transfected Dunning R3327 cells may provide a useful tool to study the effects of hyperthermia, radiation, and chemotherapeutic agents on the cellular stress response and in the establishment of HSP70 as a marker of cellular resistance in the multimodal treatment of prostate cancer.

Key words Dunning R3327 cells · Hyperthermia · Stable transfection · HSP70 promoter · β-Galactosidase

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Introduction

Cancer of the prostate has become the most frequent cancer in men in the United States and Europe, with an estimated incidence of over 300 000 new cases diagnosed for the United States in 1996; it is the second leading cause of death from cancer in the male population [24]. At the time of diagnosis, more than 50% of cases show evidence of advanced disease with involvement of periprostatic tissue or lymph nodes or the development of distant metastases. In patients with locally advanced prostate cancer, hyperthermia may be a powerful tool either alone or in combination with radiation [9, 18, 26, 32]; yet little is known about the optimization of therapy in terms of the most effective doses and sequencing. Because the cellular response to stress involves the synthesis of a group of heat shock proteins that confer tolerance to subsequent stresses, an understanding of the HSP response is critical to the development of rational multimodal therapies including hyperthermia, radiation, and chemotherapeutics.

The HSPs range in molecular weight from 8 to 110 kDa. The family of the 70-kDa (HSP70) proteins has been shown to be most thermosensitive [6]. The 70-kDa HSPs consist of a 73-kDa constitutive (HSP73) and a 72-kDa inducible (HSP72) protein. Although the precise function of these proteins is not yet fully understood, the thermotolerant state of cells is associated with synthesis and decay of HSP70 and their presence is necessary for cellular survival under conditions of heat stress [12, 14].

Our laboratory has previously shown that Dunning R3327 cells accumulate HSP70 in response to hyperthermia [25]. This elevation of HSP70 was associated with thermal tolerance in these cells (unpublished data). Radiation, hypoxia, and chemotherapeutic agents such as bleomycin and cisplatin have also been shown to induce the stress response, rendering cells tolerant [7, 20, 22, 27]. Thus, HSP70 is a potentially important marker of cellular resistance in hyperthermia-based multimodal

treatment approaches. Given this potential role as marker of tumor tolerance, the study of HSP70 regulation following either hyperthermia alone or in combination with chemotherapeutic agents or radiation may be critical to establish efficacious treatment combinations as well as treatment schedules. Our laboratory has developed a rapid system of HSP70 measurement through the cloning of a Dunning R3327 cell line stably transfected with a gene construct containing the *Drosophila* HSP70 promoter controlling the *E. coli* β-galactosidase enzyme. This gene construct has been well described by Amin et al. [1, 2]. These stably transfected Dunning cells provide a rapid, colorometric and semiquantitative analysis of HSP70 activation through the measurement of β-galactosidase in in vitro studies. These cells may also be used in vivo in order to study the HSP70 response using interstitial hyperthermia in combination with radiation or chemotherapeutic agents in order to establish optimal treatment schedules considering the development of cellular tolerance.

Materials and methods

Cell line and culture conditions

Dunning R3327 rat prostate adenocarcinoma cells with a moderate differentiation were used in these experiments. The cells were a generous gift from Dr. D. M. Lubaroff, Department of Urology, University of Iowa, Iowa City, IA, and have been extensively described [15]. The cell line was derived from the 130 subline of the original Dunning tumor, is of pure epithelial character, and has been shown to be hormone responsive (Dr. D. M. Lubaroff, personal communication). Cells were grown in RPMI 1640 containing 10% fetal calf serum (FCS) with 10 mM hydroxyethylpiperazine ethanesulfonic acid (HEPES) (pH 7.0), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1 mM sodium pyruvate, and were maintained at 37°C in a humidified atmosphere containing 5% CO_2/air. Transfected cells were regularly passaged in the same media additionally supplemented with 450 μg/ml G418.

Plasmids

Dunning cells were co-transfected with an HSP70 promoter/β-galactosidase hybrid gene construct (provided by R. Voellmy) and the pSV2Neo plasmid for resistance to the antibiotic G418 [28]. The HSP70 promoter/β-galactosidase construct consists of an 88-bp segment of the *D. melanogaster* HSP70 promoter linked to an *E. coli* β-galactosidase gene inserted into the plasmid pSVOd [13].

Stable transfection of Dunning R3327 cells

The transfection was performed by calcium phosphate precipitation as previously described [30]. Twenty four hours after transfection cells were subcultured into media containing 450 μg/ml G418. Cells were plated at a low density and colonies were subcloned and expanded.

Assay of β-galactosidase activity

β-Galactosidase activity was analyzed as previously described [21]. Briefly, 70% confluent cells in 25-cm^2 tissue culture flasks were rinsed twice with phosphate-buffered saline (PBS), 0.2 ml of 0.1% SDS in PBS was added and the plates were allowed to sit for 10 min. 0.8 ml PM-2 buffer (33 μM NaH_2PO_4, 66 μM Na_2HPO_4, 0.1 mM $MnCl_2$, 2 mM $MgSO_2$, 40 μM β-mercaptoethanol) was added, the solution was transferred to an Eppendorf tube, 0.2 ml of a 4 mg/ml solution of *O*-nitrophenyl-β-D-galactose (ONPG; Sigma Chemical Co., St. Louis, MO) was added, and the samples were mixed by inversion and placed in a 37°C water bath until a color change was observed. The reaction was terminated by the addition of 0.5 ml 1 M Na_2CO_3. The tubes were cleared of cellular debris by three freeze/thaws in a dry ice/ethanol bath followed by centrifugation at 10 000 × *g* for 5 min. The amount of hydrolyzed ONPG was evaluated spectrophotometrically, and the units of β-galactosidase activity were calculated as the $A_{420} \times 380$/min reaction and related to total protein.

Western analysis of HSP70 protein

Cells were collected and lysed by freeze/thaw and total cellular protein was determined using the Bradford method [4]. From each sample 15 μg was separated on a 7.5% SDS page gel and transferred to nitrocellulose. The membranes were blocked by soaking in 10% FCS, 10% bovine serum albumin (BSA) in TBS (TBS; 10 mM TRIS, 150 mM NaCl, pH 8.0) for 3 h at room temperature. The blots were rinsed once in TBS with 0.5% Tween-20, 3 times in TBS, then probed for 1 h with a monoclonal antibody specific for the inducible 72-kDa HSP (C92, StressGen Biotechnologies Co., Victoria, BC, Canada). The blot was incubated with a secondary alkaline phosphatase linked antibody and was re-rinsed, and then incubated in 0.45 mM 5-bromo-4-chloro-3-indolyl phosphate (BCIP, Sigma, St. Louis, MO) and 0.27 mM nitroblue tetrazolium (NBT, Fisher Scientific, Fair Lawn, NJ), in AP buffer (100 mM TRIS, 100 mM NaCl, and 5 mM $MgCl_2$, pH 9.5) until the bands developed.

Southern analysis of gene incorporation

Genomic DNA was collected and the Southern analysis performed as previously described [5]. Briefly, 10 μg DNA from both untransfected Dunning cells and the A1 clone was either cut with *Cla*I or left uncut and run overnight on 0.7% agarose gel. The gel was photographed after ethidium bromide staining to determine equal loading and then transferred to a nylon membrane (Amersham Life Science, Arlington Heights, IL). The membrane was air dried and fixed by exposure to UV light.

Probe labeling and hybridization

A *Bam*HI to *Xho*I restriction fragment of D88, which specifically hybridizes with the β-galactosidase gene, was isolated from an agarose gel, and probes were generated by using a random-primed DNA-labeling kit (Boehringer Mannheim, Indianapolis, IN) and ^{32}P-dCTP. The blot was pre-hybridized in the hybridization solution [1 M NaCl, 50 mM TRIS-HCl (pH 7.4), 40% formamide, 10% dextran sulfate, and 1% SDS] for 1 h at 42°C. The ^{32}P-labeled probe was added and the blot was incubated at 42°C overnight with gentle shaking. The blot was rinsed twice with 2 × SSC/0.1% SDS [20 × SSC: 3 M sodium chloride, 300 mM sodium citrate (pH 7.0)] at room temperature and twice with 0.1% SSC/0.1% SDS at 42°C before exposure to X-ray film.

Heat stress

Cells were grown to 70% confluence in 25-cm^2 tissue culture flasks and either maintained in a 37°C incubator or placed into a precision water bath set at 43.5°C (±0.1°C) for 1 h in a humidified atmosphere containing 5% CO_2/air. Temperature was monitored by placing a copper constantin thermocouple into separate 25-cm^2 tissue culture flasks containing media. After heating, the cells were then returned to the 37°C incubator and collected 4, 8, 12, 24, or 48 h later. For experiments studying thermotolerance, cells were

conditioned with a 43°C heat stress for 1 h with 16 h recovery followed by a second heat stress of 43.5°C for 1 h. Cells were then prepared for colony formation assessment.

Colony formation assay

Following treatment cells were trypsinized, counted with a hemocytometer, plated in triplicate in 25-cm^2 culture flasks and cultured for 7 days in a humidified atmosphere containing 5% CO_2/air. After 7 days of growth, the cells were stained with 1% (w/v) methylene blue for 10 min and colonies containing at least 50 cells were scored. The surviving fraction was determined as the percentage of the number of colonies/plated cells in each treated group versus the number of colonies/plated cells in the control group of each experiment. All clonogenic experiments were repeated at least twice.

Results

The initial cell clone demonstrating G418 resistance and β-galactosidase activity following a 43.5°C heat stress was termed A1. In order to determine whether the cloning procedure had resulted in the incorporation of the HSP70 promoter β-galactosidase gene into the Dunning cell genome, Southern blot analysis was performed of genomic DNA extracts using a ^{32}P probe containing the *E. coli* β-galactosidase gene. As demonstrated in Fig. 1, DNA from transfected Dunning cells shows evidence of β-galactosidase incorporation within the genome. Dunning cells that were not transfected with the HSP70 promoter β-galactosidase gene showed no evidence of recognition of the β-galactosidase probe.

To determine the correlation between β-galactosidase activity and the accumulation of cellular HSP70 following heat stress, cells of the A1 clone were exposed to a 43.5°C ambient temperature for 1 h, then evaluated for both β-galactosidase activity and the presence of inducible HSP70 by Western blotting. Data obtained from transfected cells revealed a time-dependent increase in β-galactosidase activity following heat treatment beginning at 4 h following heat exposure, reaching a maximum at 12 and 16 h, and returning to baseline after 48 h post-exposure (Fig. 2). HSP70 protein accumulation by Western blot analysis demonstrated an identical time course (Fig. 3). The first elevations in inducible HSP70 were seen at 4 h post heat treatment, reached a maximum at 12 and 16 h, and returned to baseline after 48 h. The transfection procedure had no effect on the cellular stress response in that transfected and untransfected Dunning cells showed no difference in HSP70 protein accumulation following hyperthermic stress.

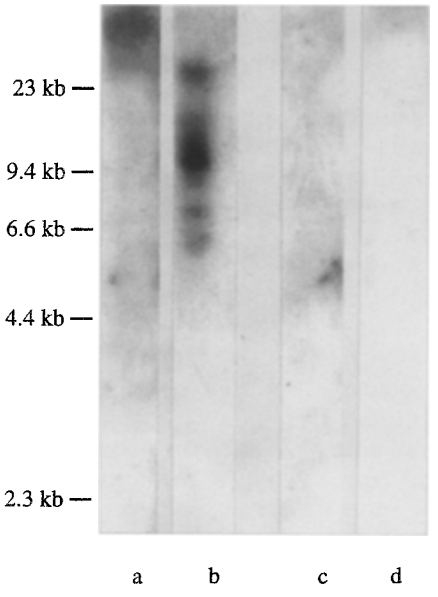

Fig. 1 Southern blot analysis with a ^{32}P-labeled probe hybridizing with the β-galactosidase gene. A1, the transfected clone of Dunning cells (*lanes a, b*) shows evidence of β-galactosidase gene incorporation compared to untransfected control cells (*lanes c, d*). (*lanes a, c* represent undigested DNA, *lanes b, d* represent *Cla*I-digested DNA)

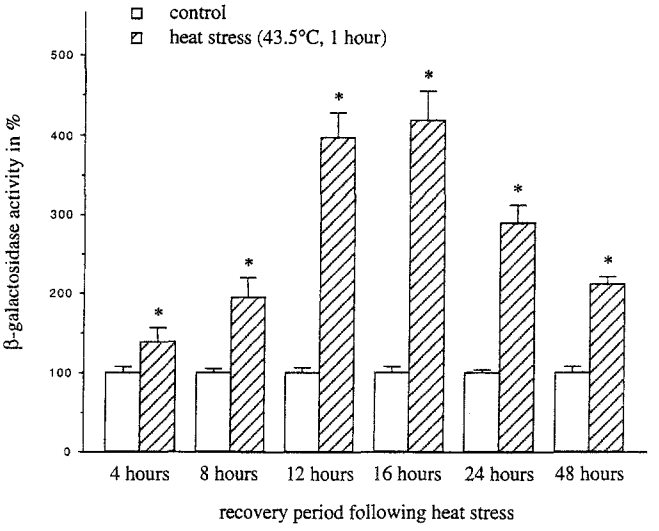

Fig. 2 β-Galactosidase activity/protein (expressed as a percentage for each unheated control) of the clone A1 following a 1-h heat shock at 43.5°C at different time points during recovery. * indicates significance at $P < 0.05$ by analysis of variance, $n \geq 4$ in each group; all values are shown as means ± SD

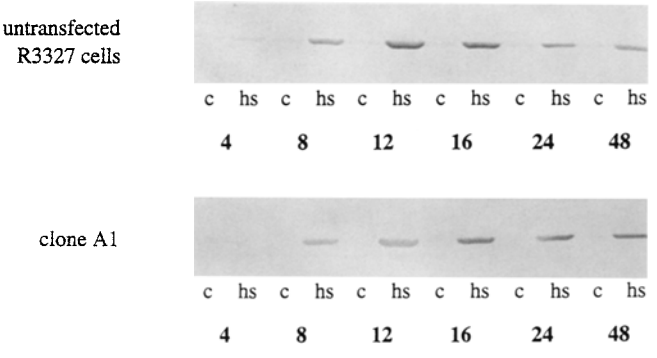

Fig. 3 Western blots of HSP70 protein from untransfected Dunning R3327 cells and the transfected clone A1 following a 1-h heat shock (*hs*) at 43.5°C compared to unheated controls (c). *Numbers* indicate recovery time in hours

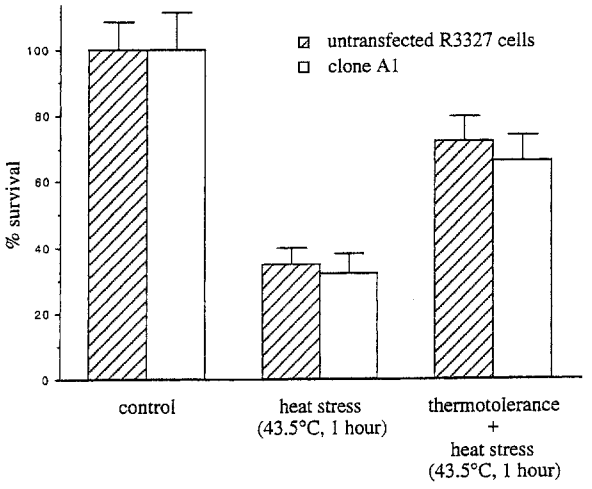

Fig. 4 Colony formation ability of Dunning R3327 cells and the transfected clone A1.[6] The data reveal that the thermal sensitivity following a 43.5°C heat stress for 1 h is unchanged in transfected cells. When cells were conditioned with a 43°C heat stress the development of thermotolerance was similar in both, transfected and untransfected cells. * indicates significance at $P < 0.05$ by analysis of variance; $n \geq 4$ in each group; all values are shown as means ± SD

The clone A1 was also tested for the stability of the β-galactosidase signal following a similar heat stress (43.5°C, 1 h with 16 h recovery) at extended passages. Data revealed that the heat response of β-galactosidase activity remained stable up to 25 passages following transfection (results not shown). For experiments cells at passages from 5 up to 20 were used. As shown by the colony formation data in Fig. 4, the transfection did not alter the sensitivity of the clone A1 to a 43.5°C heat stress or the ability to develop thermotolerance (following a conditioning heat stress) compared to untransfected Dunning cells.

Discussion

We have successfully developed a stable clone of Dunning R3327 cells with a gene construct containing the *E. coli* β-galactosidase gene driven by the HSP70 *Drosophila* promoter. The Southern analysis using a ^{32}P-labeled probe which recognizes the β-galactosidase gene documented the incorporation of the HSP70 β-galactosidase hybrid into the genomic DNA of the cells from the clone A1. Hyperthermic exposure of these cells caused a more than threefold increase in β-galactosidase activity compared to transfected but unheated controls. The time course of heat-induced β-galactosidase elevation closely paralleled the time course of the endogenous HSP70 protein accumulation in these cells following heat stress. These data demonstrate that the measurement of β-galactosidase activity accurately predicts endogenous HSP70 protein accumulation following heat stress.

The transfection procedure did not alter the stress response of the clone A1 in that there was no difference in heat-induced endogenous HSP70 production between the stably transfected clone A1 and untransfected Dunning R3327 cells (Fig. 3). Further heat sensitivity and the ability to develop thermotolerance remained unchanged.

Hybrid genes in which the expression of bacterial β-galactosidase was controlled by the *Drosophila* HSP70 promoter have been successfully used as a rapid assay to understand HSP gene activation and regulation in eukaryotic cells [3]. Gene constructs using HSP70 promoter mutants with 90 up to 1140 bp strongly expressed β-galactosidase activity in *D. melanogaster* and COS-1 (African green monkey kidney) cells [1, 20], while deletion studies have shown insufficient or no β-galactosidase activity in these cells following a heat stress [1, 2]. Anathan and coworkers observed an increased β-galactosidase signal as a measure of HSP70 promoter activation similar to the native HSP expression of cells following exposure to abnormal proteins, for example, amino acid analogs indicating that the hybrid gene exactly reflects endogenous HSP promoter activity [3].

There is evidence in the literature for both transcriptional and post-transcriptional regulation of HSP70. Our model does not directly assess post-transcriptional events in HSP70 regulation, but does provide an assay system that accurately reflects HSP70 accumulation following heat stress. Whether post-transcriptional events would further enhance the response is unknown. The HSP70 β-galactosidase gene construct has also been shown to respond to the chemotherapeutic agent bleomycin in transiently transfected COS-1 cells [20]. Similarly, transient transfections of A549 (human lung cancer) cells have revealed that certain oxidant stresses also result in the induction of the HSP70 as measured by elevated β-galactosidase activity [31].

These data indicate that the clone A1 of Dunning R3327 may provide an easy system to study HSP70 regulation following other therapeutic agents, for example, radiation and chemotherapeutics, allowing the establishment of adequate dosing and sequencing of multimodal therapies. This information may be crucial to optimize tumor response, since studies in yeast have shown that tolerance to both radiation and heat occurs following a hyperthermic stress [17]. In addition, antineoplastic drugs such as cisplatin and Adriamycin, which are of clinical value in the treatment of advanced prostate cancer [16, 19, 29], are known to have synergistic cytotoxic effects with hyperthermia.

Several studies have explored the interaction between chemotherapeutic agents, hyperthermia, and HSP regulation and revealed that a chemotherapeutic-resistant tumor cell state is often associated with the overexpression of HSPs [8, 10, 11, 22, 23] and combined with thermal tolerance [22]. These data strongly support the theory of the involvement of certain HSPs in the development of tumor cell resistance in hyperthermia-based multimodal treatment approaches. Our system of β-galactosidase as a rapid and quantifiable marker of HSP70 promoter induction may be a useful cellular

model to further investigate effective doses and sequencing patterns in multimodal treatments of locally advanced prostate cancer.

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