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Active heat shock transcription factor 1 supports migration of the melanoma cells via vinculin down-regulation

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ABSTRACT

Heat shock transcription factor 1 (HSF1), the major regulator of stress response, is frequently activated in cancer and has an apparent role in malignant transformation. Here we analyzed the influence of the over-expression of a constitutively active transcriptionally-competent HSF1 mutant form on phenotypes of mouse and human melanoma cells. We observed that the expression of active HSF1 supported anchorage-independent growth *in vitro*, and metastatic spread in the animal model *in vivo*, although the proliferation rate of cancer cells was not affected. Furthermore, active HSF1 enhanced cell motility, reduced the adherence of cells to a fibronectin-coated surface, and affected the actin cytoskeleton. We found that although the expression of active HSF1 did not affect levels of epithelial-to-mesenchymal transition markers, it caused transcriptional down-regulation of vinculin, protein involved in cell motility, and adherence. Functional HSF1-binding sites were found in mouse and human *Vcl*/*VCL* genes, indicating a direct role of HSF1 in the regulation of this gene. An apparent association between HSF1-induced down-regulation of vinculin, increased motility, and a reduced adherence of cells suggests a possible mechanism of HSF1-mediated enhancement of the metastatic potential of cancer cells.

1. Introduction

Heat Shock Transcription Factor 1 (HSF1) is the major regulator of cellular response to stress, which is activated by various environmental and pathophysiological stimuli [2]. It is the primary transcriptional regulator of genes encoding for heat shock proteins (HSPs), which function as molecular chaperones and play an important role in the regulation of protein homeostasis and cell survival during proteotoxic stress. Moreover, HSPs mediate cytoprotection by the direct inhibition of key steps of programmed cell death [3]. Beyond the regulation of *HSPs* expression, HSF1 binds a broad array of non-*HSP* genes [18,29,37]. This property of HSF1 seems to be important in processes associated with development and growth [1,32], fertility [26,39,42], and longevity [30], which are not related directly with the HSF1-dependent regulation of HSPs.

A high level of HSF1 expression was observed in a broad range of cancer cell lines and human tumors including colorectal cancer, breast cancer, oral squamous cell carcinoma, hepatocellular carcinoma, multiple myeloma, and glioma [41]. Increased HSF1 expression has been associated with a reduction in the survival rate and was proposed as an independent prognostic factor for overall survival in patients with estrogen receptor-positive breast cancer [33] and hepatocellular carcinoma [8]. HSF1 could also change the sensitivity of cancer cells to cytotoxic treatment [15,40]. However, the specific role of HSF1 activity in malignant cells is not fully known. HSF1 is not a classical oncogene or tumor suppressor but rather interferes with many metabolic processes supporting tumor growth, which was called a "non-oncogene addiction" [35]. It was previously shown that HSF1 activation diminishes cell cycle checkpoints leading to aneuploidy [21] and influences RAS/MAPK and cAMP/PKA signal transduction pathways essential for malignant growth [7]. Furthermore, MEFs derived from *Hsf1*$^{-/-}$ animals were unable to form colonies in soft agar [16] and were deficient in migration induced by basal and epidermal growth factors [28]. The motility of bone marrow cells isolated from *Hsf1*$^{-/-}$ mice was also reduced [17]. The above-mentioned data suggests that HSF1 can affect the ability of cells to migrate. A high migration potential is thought to be a prerequisite for tumor cell invasion and spread, hence suggesting the involvement of HSF1 in the regulation of cancer metastasis. Importantly, HSF1 was found among six metastasis-promoting genes in malignant melanomas [34]. Its ability to promote migration and invasion was also observed in hepatocellular carcinomas [8]. Nevertheless, the exact

Abbreviations: EMT, epithelial to mesenchymal transition; HS, heat shock; HSF1, heat shock transcription factor 1; HSPs, heat shock proteins; MEFs, mouse embryonic fibroblasts

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role of HSF1 in the regulation of cancer cell growth and motility still remains unclear.

In this study, we aimed to determine the role of HSF1 in tumor growth and in the ability of cells to migrate. Melanoma cell lines (mouse B16F10 and human WM793B and 1205Lu) with modified levels of HSF1 activity were established and tested for different migration-related processes. We found that active HSF1 can support cell motility via a mechanism mediated by down-regulation of vinculin and an influence on the actin cytoskeleton organization.

2. Materials and methods

2.1. Experimental model

Melanoma cell lines: B16F10, WM793B and 1205Lu were purchased from the American Type Cell Culture Collection (ATCC, Manassas, VA). Cell culture and heat shock treatment were performed as described in details elsewhere [40].

2.2. DNA constructs and transfections

The aHSF1 (hHSF1ΔRD) construct, containing the active form of human HSF1 (with deletion of amino acid 221–315) driven by the human β-actin promoter, was kindly provided by Dr. A. Nakai [26]. The aHSF1 sequence was re-cloned into the pLNCX2 retrovirus expression vector (Clontech) downstream of the CMV promoter [14]. The target sequences for knock-down of mouse *Hsf1* gene were as follows: HSF1-1 (1856–1876, NM_008296.2)—5′ GCTGCATACCTGCTGCCTTTA; and HSF1-2 (341–359, NM_008296.2)—5′AGCACAACAACATGGCTAG. Stably transfected B16F10, WM793B and 1205Lu cells were obtained as described in details elsewhere [40].

2.3. Thermotolerance test

Logarithmically growing cells were thermally preconditioned by placing them in a water bath at a temperature of 42 °C for 1 h, then cells were allowed to recover in a CO_2 incubator at 37 °C for 6 h before a subsequent severe heat shock (1 h at 45 °C for B16F10 cells, 1 h at 48 °C for 1205Lu cells, or 30 min at 49 °C for WM793B cells). Alternatively, cells were subjected to a severe heat shock without preconditioning (condition of severe heat stress was established experimentally to achieve a cell survival rate which was higher than 50%). After heat shock cells were allowed to recover at 37 °C for 18 h, then harvested, stained with propidium iodide and analyzed on a FACS Canto cytometer (Becton Dickinson). Cell survival rate was calculated in relation to untreated controls; all experiments were performed in triplicate at least.

2.4. Proliferation test

Cells (2×10^4 cells per well) were seeded and cultured in 12-well plates. At the indicated time cells were washed with PBS, fixed in cold methanol, and rinsed with distilled water. Cells were stained with 0.1% crystal violet for 30 min, rinsed with distilled water extensively, and dried. Cell-associated dye was extracted with 1 ml of 10% acetic acid. Aliquots (200 μl) were transferred to a 96-well plate and the absorbance was measured at 595 nm (Synergy2, BioTek). Values were normalized to the optical density on day 0; all experiments were performed in triplicate at least.

2.5. Anchorage-independent growth

B16F10 cells (2×10^3 per well, in 1 ml of growth medium containing 0.35% noble agar, Difco, Becton Dickinson) were seeded on top of a solid growth medium containing 1% noble agar (1 ml) in a 12-well plate. Cells were covered with 300 μl of growth medium, which was supplemented every 3 days. After 21 days colonies were stained with 0.1% crystal violet and counted. All experiments were performed in triplicate at least.

2.6. Trans-well migration test (Boyden Chamber Assay)

Transwell chambers (with 8-μm pore size membrane, Becton Dickinson) were coated with fibronectin (10 μg/ml, Becton Dickinson). Cells (5×10^4 of B16F10 or 1205Lu, and 2.5×10^4 of WM793B) were suspended in a Hepes-buffered serum-free medium containing 0.1% BSA, seeded in the top of the chambers, and placed in the wells containing medium supplemented with 10% fetal bovine serum. After 4 h (B16F10 and 1205Lu) or 2 h (WM793B) the inserts were washed with PBS, fixed with cold methanol, rinsed with distilled water, and stained with 0.1% crystal violet for 30 min. The cells on the upper surface of the inserts were gently removed with a cotton swab. Cells that migrated onto the lower surface were counted under a microscope in five random fields; all experiments were performed in triplicate at least.

2.7. In vivo animal experiments

B16F10 cells (2×10^5 cells per mouse) were injected subcutaneously into 6–8-week-old C57BL/6 females (experimental group $n = 6$). Tumor growth was evaluated daily (for 8 days starting from tumor size ~10 mm^3) by caliper measurement in two perpendicular dimensions. Tumor volume was calculated using the formula: volume (mm^3) = 0.52 × (width)^2 × length [44]. To study the ability of B16F10 cells to metastasize, cells (2×10^5 cells per mouse) were injected into the tail vein (experimental group $n = 10$). Ten days after injection, mice were sacrificed by cervical dislocation. The lungs were collected and fixed in Bouin's solution. Metastasis foci were counted. All animal procedures were conducted in accordance with the recommendation of the Polish Council on Animal Care and were approved by the Local Committee of Ethics and Animal Experimentation at the Medical University of Silesia in Katowice, Poland (Decision No 77/2008 and 42/2012). The animals were maintained under controlled environmental conditions with a 1:1 light:darkness cycle, and were provided with unrestricted access to food and tap water.

2.8. Ki-67 index

Ki-67 immunostaining with polyclonal anti-rabbit antibody (1:1000; ab66155, Abcam) was performed on 5 μm-sections of paraformaldehyde-fixed (4% in PBS, overnight at 4 °C) and paraffin-embedded B16F10 tumors. An antigen retrieval step in 0.01 M citrate buffer pH 6.0 was performed before the immunohistochemistry procedure. An ImmPRESS Anti-Rabbit Ig (peroxidase) Polymer Detection Kit (Vector Labs, Burlingame, CA, USA) was used according to the manufacturer's guideline. DAB (3′,5′-diaminobenzidine) was used as a chromogen for visualization of immunohistochemical reactions and hematoxylin was used for nuclei counterstaining. Negative controls for specificity of the staining were performed by omitting the primary antibody. The Ki-67 index was determined by manual counting of >2000 cells from each section and shown as the relative number of nuclei showing positive staining.

2.9. RT^2 Profiler PCR array and quantitative RT-PCR

Expression of motility-related genes was assessed using the Mouse Cell Motility RT^2 Profiler PCR Array (SABiosciences, Frederick, USA). Total mRNA was isolated using a RNeasy kit (Qiagen) and converted into cDNA using a RT^2 First Strand Kit (SABiosciences, Frederick, USA). The cDNA was added to the RT^2 SYBR Green qPCR Master Mix (SABiosciences, Frederick, USA) and aliquotted on the Mouse Cell Motility PCR arrays. All steps, including analysis of PCR data, were carried out according to the manufacturer's protocol using the CFX96™ Bio-Rad Real-time System. Gene-specific quantitative RT-PCR was performed