

Targeting the Heat Shock Factor 1 by RNA Interference: A Potent Tool to Enhance Hyperthermochemotherapy Efficacy in Cervical Cancer

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Abstract

Carcinoma of the uterine cervix is one of the highest causes of mortality in female cancer patients worldwide, and improved treatment options for this type of malignancy are highly needed. Local hyperthermia has been successfully used in combination with systemic administration of cisplatin-based chemotherapy in phase I/II clinical studies. Heat-induced expression of cytoprotective and antiapoptotic heat shock proteins (HSP) is a known complication of hyperthermia, resulting in thermotolerance and chemoresistance and hindering the efficacy of the combination therapy. Heat shock transcription factor 1 (HSF1) is the master regulator of heat-induced HSP expression. In the present report, we used small interfering RNA (siRNA) to silence HSF1 and to examine the effect of HSF1 loss of function on the response to hyperthermia and cisplatin-based chemotherapy in HeLa cervical carcinoma. We have identified the 322-nucleotide to 340-nucleotide HSF1 sequence as an ideal target for siRNA-mediated HSF1 silencing, have created a pSUPER-HSF1 vector able to potently suppress the *HSF1* gene, and have generated for the first time human cancer cell lines with stable loss of HSF1 function. We report that, although it surprisingly does not affect cancer cell sensitivity to cisplatin or elevated temperatures up to 43°C when administered separately, loss of HSF1 function causes a dramatic increase in sensitivity to hyperthermochemotherapy, leading to massive (>95%) apoptosis of cancer cells. These findings indicate that disruption of HSF1-induced cytoprotection during hyperthermochemotherapy may represent a powerful strategy to selectively amplify the damage in cancer cells and identify HSF1 as a promising therapeutic target in cervical carcinoma. (Cancer Res 2006; 66(15): 7678-85)

Introduction

The heat shock response (HSR) is a fundamental cellular defense mechanism against the deleterious effects of physiologic and environmental stresses, which provoke cell damage due to protein misfolding, degradation, and insoluble aggregation (1). The HSR is regulated by a family of transcription factors known as heat shock factors (HSF; ref. 2). Among three functionally different HSFs in humans, HSF type 1 (HSF1) mediates signaling of stress-induced

stimuli, such as elevated temperatures. HSF1 is generally found in the cytoplasm as an inert monomer lacking transcriptional activity; both DNA-binding and transcriptional transactivation domains are repressed through intramolecular interactions and constitutive serine phosphorylation (3). On exposure to heat shock and other types of stress, HSF1 DNA-binding activity is derepressed. Subsequently, monomers oligomerize to a trimeric state and form stress-induced intranuclear granules, become phosphorylated at serine residues, and bind to heat shock elements (HSE) located upstream of heat shock-responsive genes, switching on stress-induced transcription (4). Heat shock gene promoters that contain HSEs include the molecular chaperones of the heat shock protein (HSP) families HSP70 and HSP90, of HSP27, and other proteins of the network. HSF-binding sites are also present in the promoters of the superoxide dismutase and the multidrug resistance genes (5, 6).

HSP expression is tightly controlled in normal human cells, whereas it is often dysregulated in tumor cells. Aberrant expression of HSP90, HSP70, and HSP27 either individually or in combination has been widely reported in human malignant tumors of various origins (7–9) and has been linked to cancer resistance to apoptosis induced by chemotherapy and radiation therapy (10). On the other hand, hyperthermia is one of the modalities used in the clinical setting to treat various forms of malignancies and has been proven to be especially effective in combination with radiotherapy and chemotherapy in different types of cancer, including carcinoma of the uterine cervix (11, 12). For cervical carcinoma, local hyperthermia has been successfully used in combination with systemic administration of cisplatin-based chemotherapy in phase I/II clinical studies (13).

Because of the substantial technical improvements recently achieved (11), the clinical application of heat as a coadjuvant in cancer treatment is gaining new interest. However, one of the main complications associated with the use of hyperthermia in cancer treatment is the induction of the cellular stress response, which prevents irreversible inactivation of proteins and blocks apoptotic signaling, enhancing cell survival (10, 14–17). Therefore, strategies that inhibit the HSR would be useful to sensitize cancer cells to therapies that use hyperthermia as a modality of treatment.

HSF1, being the main HSR regulator, seems to be a desirable target to inhibit the stress response in cancer cells. By using small interfering RNA (siRNA)-mediated silencing, we attempted to obtain a HSF1 loss-of-function phenotype in HeLa cervical carcinoma to examine the effect of HSF1 silencing on the response to hyperthermia and cisplatin-based chemotherapy. Herein, we report that loss of HSF1 function, although it surprisingly does not affect cancer cell sensitivity to cisplatin chemotherapy or to elevated temperatures when administered separately, results in a dramatic increase in sensitivity to apoptosis induced by cytostatic doses of cisplatin associated with hyperthermia.

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Materials and Methods

Cell culture, treatments, and transfections. HeLa cells (American Type Culture Collection, Manassas, VA) were grown at 37°C in a 5% CO₂ atmosphere in DMEM supplemented with 10% FCS, 2 mmol/L glutamine, and antibiotics (Life Technologies, Inc., Paisley, Scotland). HeLa cells stably transfected with pSUPER/pcDNA3 and pSUPER-HSF1i were maintained in DMEM supplemented with 10% FCS in the presence of G-418 (400 µg/mL). Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to MTT formazan conversion assay (Sigma-Aldrich, St. Louis, MO). For MTT assay, reduced MTT (formazan) was extracted from cells by adding 100 µL acidic isopropanol containing 10% Triton X-100, and formazan absorbance was measured in an ELISA microplate reader at two different wavelengths (540 and 690 nm). Cisplatin [*cis*-diammineplatinum(II) dichloride, Sigma Chemical Co., St. Louis, MO] was dissolved in 0.9% NaCl immediately before use. For heating procedures, cells were kept at different temperatures (ranging from 41°C to 45°C) in an incubator in a 5% CO₂ atmosphere. For combined treatments, hyperthermia was administered 2 hours after exposure to cisplatin to mimic previously reported clinical setting conditions (13). All transfections were done using LipofectAMINE Plus reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocols.

Vector construction. The procedure for preparing constructs coding for siRNA was described previously (18). Among different HSF1 target sequences examined, the 19-nucleotide gene-specific sequence spanning from nucleotides 322 to 340 downstream of the gene transcription start site was selected to suppress HSF1 gene expression. After BLAST analysis, to ensure that there was no significant sequence homology with other human genes, the selected sequence was inserted into a *Bgl*II/*Hin*dIII-cut pSUPER vector to generate the pSUPER-HSF1i vector. The pSUPER-p53i vector was generated as described above using the sequence reported in ref. 18. The pSUPER-GFPi vector was a gift from Dr. Antonio Costanzo (University of Rome Tor Vergata, Rome, Italy). Epitope Flag-tagged HSP27 vector was a gift from Dr. Kyu-Jin Park (University of Texas, Southwestern Medical Center, Dallas, TX). The pH2.3/hsp70 and pKNI-3/hsp90 vectors were kindly provided by Dr. Richard I. Morimoto (Northwestern University, Evanston, IL). To generate the Flag-tagged hsp70-pcDNA3 vector, the human *HSP70* gene was amplified from the pH2.3/hsp70 vector using the following primers: 5'-GGCGGATCCACCATGGCCAAAGCCGCGGCAGTC-3' and 5'-TGC-GGTTCGACCTACTTATCGTCGTCATCCTTGTAAATCATCTACCTCCTCAATGGTGGG-3'. The PCR product was digested with *Bam*HI and *Sal*I and inserted into a *Bam*HI/*Sal*I-cut pcDNA3 vector. For construction of the Flag-tagged hsp90-pcDNA3 vector, the human *HSP90* gene was amplified from the pKNI-3/hsp90 vector using the following primers: 5'-TTTGCTAGCCCTGAGGAAGTGCACC-3' and 5'-AAACTCGAGCTAATCG-ACCTTCCATG-3'. The PCR product was digested with *Nhe*I and *Xba*I and inserted in frame into a *Nhe*I/*Xba*I-cut pcDNA3 vector containing the Flag-tagged sequence at the COOH terminus. All constructs were confirmed by DNA sequence analysis.

Generation of stable HSF1-silenced lines. To obtain HeLa cell lines stably expressing siRNAs, the pSUPER or pSUPER-HSF1i vectors were cotransfected with the pcDNA3 vector, and stable integrants were selected by using G-418 (1 mg/mL) starting 72 hours after transfection. After 15 days in selective medium, two pools, referred as HeLa-pSUPER-p and HeLa-HSF1i-p, were isolated. The HeLa-HSF1i-p was subjected to further cloning procedures by the limiting dilution method. After 2 months, three clones presenting a >95% reduction in HSF1 protein levels compared with control (HeLa-HSF1i clones 3, 16, and 31) were isolated. All stable cell lines obtained were cultured in DMEM supplemented with G-418 (400 µg/mL). The G-418 selective pressure was removed 24 hours before experimental procedures.

Confocal microscopy. Cells grown on coverslips were fixed with 4% paraformaldehyde and permeabilized in 0.1% Triton X-100-PBS for 10 minutes. After blocking with 0.2% bovine serum albumin, cells were incubated with rat anti-HSF1 monoclonal antibody (Stressgen Biotechnologies, Victoria, British Columbia, Canada) followed by decoration with fluorescein-conjugated anti-rat IgG. Images were acquired on a Leica confocal microscope (TCS 4D system, 100× objective; Leica, Nussloch, Germany).

Electrophoretic mobility shift assay. Whole-cell extracts (15 µg protein) prepared after lysis in high-salt extraction buffer (19) were incubated with a ³²P-labeled HSE DNA probe followed by analysis of DNA-binding activity by electrophoretic mobility shift assay (EMSA). Binding reactions were done as described (20). Complexes were analyzed by non-denaturing 4% PAGE. Quantitative evaluation of HSF-HSE complex formation was determined by Typhoon 8600 imager with the use of ImageQuant (Molecular Dynamics, Amersham Pharmacia Biotech, Piscataway, NJ).

Western blot analysis. Equal amounts of protein (35 µg/sample) from whole-cell extracts were separated by SDS-PAGE and blotted to nitrocellulose. After blocking with 5% skim milk solution, membranes were incubated with rabbit polyclonal anti-HSF1, anti-HSF2 (kindly provided by Dr. Richard I. Morimoto), anti-poly(ADP-ribose) polymerase (PARP), anti-p65, anti-HSP90, and anti-HSP27 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) or monoclonal anti-HSP70 inducible isoform (Stressgen Biotechnologies) and anti-β-actin (Sigma) antibodies followed by decoration with peroxidase-labeled anti-rabbit or anti-mouse IgG, respectively (Super Signal detection kit, Pierce, Rockford, IL). Quantitative evaluation of proteins was determined by VersaDoc 1000 analysis using the Quantity One software program (Bio-Rad Laboratories, Hercules, CA).

RNA analysis. RNA from HeLa cells transiently transfected with pSUPER-HSF1i or from HeLa-HSF1i clones was extracted as described previously (21). RNA (30 µg) was loaded on a 11% denaturing polyacrylamide gel and separated and blotted as described (22). Membranes were probed with a ³²P-labeled sense 19-nucleotide HSF1 target oligonucleotide and visualized by PhosphorImager. 5'-Fluorescein-labeled oligos were used as markers.

Analysis of cell cycle and apoptosis. For cell cycle studies, HeLa-pSUPER-p and HeLa-HSF1i cells were trypsinized and fixed for 45 minutes in 70% ice-cold ethanol. After repeated washings, cells were stained with a solution containing 100 µg/mL RNase A and 40 µg/mL propidium iodide for 30 minutes at room temperature and analyzed on a FACScan (Becton Dickinson Labware, Mountain View, CA) with laser excitation at 488 nm. The percentage of cells in each stage of the cell cycle was determined using CellQuest software (Becton Dickinson Labware). For DNA staining, cells were fixed with methanol and incubated with 5 µg/mL 4',6-diamidino-2-phenylindole (DAPI) for 30 minutes in the dark. Floating cells were also fixed and returned to the respective wells. Cells were analyzed using an Olympus microscope (Olympus Corporation, Tokyo, Japan) at 420 nm. Cells exhibiting morphologic features of apoptosis, including chromatin condensation and nuclear fragmentation, were counted in six to eight randomly selected fields (23). Approximately 500 nuclei were examined for each sample. The results are expressed as the number of apoptotic nuclei over the total number of nuclei counted.

Results

Selective inhibition of HSF1 gene expression by siRNA-mediated silencing. To suppress HSF1 gene expression in HeLa cells, we first identified a 19-nucleotide gene-specific sequence spanning from 322 to 340 downstream of the gene transcription start site and then generated a pSUPER vector expressing a specific siRNA targeting this sequence in the *HSF1* gene (pSUPER-HSF1i). Subconfluent HeLa cells were either mock transfected or transfected with empty pSUPER, pSUPER encoding siRNA against green fluorescent protein (GFP; pSUPER-GFPi), pSUPER encoding siRNA against p53 (pSUPER-p53i), or pSUPER-HSF1i vectors. After 72 hours, HSF1 protein levels were determined in whole-cell extracts of transfected cells by Western blot analysis. As shown in Fig. 1A, HSF1 protein levels were dramatically reduced in cells transfected with pSUPER-HSF1i but not in cells transfected with all other vectors, identifying the 322- to 340-nucleotide HSF1 sequence as an ideal target for siRNA-mediated HSF1 silencing.

The pSUPER-HSF1i vector was then used to induce HSF1 stable suppression in HeLa cells. Subconfluent HeLa cells were cotransfected with pcDNA3 and either the empty pSUPER or the

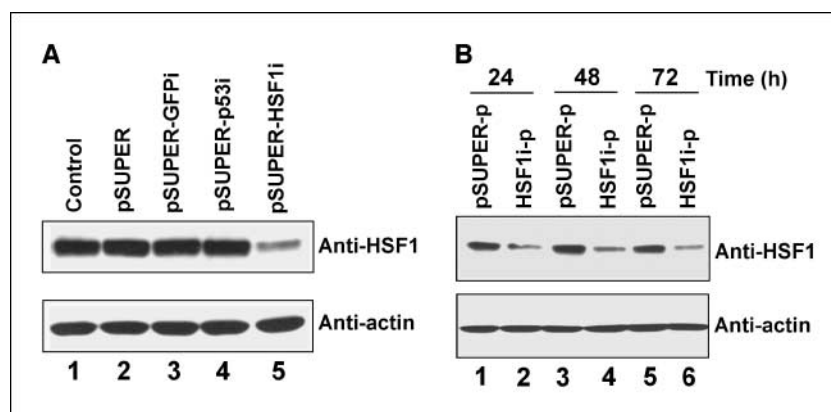


Figure 1. Silencing of HSF1 by transient and stable transfection in HeLa cells. *A*, HeLa cells were transfected with the indicated pSUPER-RNAi vectors. *Top*, after 72 hours, HSF1 levels were determined by Western blot analysis in whole-cell extracts from mock-transfected cells (*control*, lane 1) or cells transfected with empty pSUPER (*pSUPER*, lane 2), pSUPER encoding siRNA against GFP (*pSUPER-GFPi*, lane 3), pSUPER encoding siRNA against p53 (*pSUPER-p53i*, lane 4), or pSUPER encoding siRNA against HSF (*pSUPER-HSF1i*, lane 5) vectors; *bottom*, levels of β -actin were determined in the same samples as loading control. *B*, HeLa cells were cotransfected with pcDNA3 and either the empty pSUPER or the pSUPER-HSF1i vectors, and stable integrants were selected by using G-418 (see Materials and Methods for details). *Top*, HSF1 levels were determined by Western blot analysis in whole-cell extracts from pools of HeLa-pSUPER (*pSUPER-p*, lanes 1, 3, and 5) and HeLa-HSF1i (*HSF1i-p*, lanes 2, 4, and 6) cells at different times after plating; *bottom*, levels of β -actin were determined in the same samples as loading control. Western blot analysis of one representative experiment of three with identical results.

pSUPER-HSF1i vectors; after 72 hours, stable integrants were selected by using G-418. After 15 days in selective medium, resistant pools of pSUPER/pcDNA3-cotransfected (HeLa-pSUPER-p) and pSUPER-HSF1i/pcDNA3-cotransfected (HeLa-HSF1i-p) cells were obtained.

HSF1 levels in HeLa-pSUPER-p and HeLa-HSF1i-p pools were analyzed by Western blotting at 24, 48, and 72 hours after plating and quantified by densitometric analysis. As shown in Fig. 1*B*, HSF1 protein levels in HeLa-HSF1i-p cells were reduced by $\sim 70\%$ compared with HeLa-pSUPER-p cells at all times tested. The fact that HSF1 protein levels were kept remarkably low during cell growth for at least 72 hours indicates that siRNA-mediated HSF1 silencing is not influenced by cell cycling in this model.

Isolation and characterization of HeLa clones with stable loss of HSF1 function. As described above, HSF1 levels are substantially reduced in HeLa-HSF1i-p cells; however, as reported for several other siRNA targets, HSF1 inhibition obtained by RNA interference (RNAi) is not complete. In the attempt to generate stable HeLa phenotypes with loss of HSF1 function, HeLa-HSF1i-p cells were subjected to cloning procedures by the limiting dilution method. After 2 months, 60 clones were isolated and analyzed by Western blot for HSF1 protein levels. HSF1 reduction varied from 50% to $>95\%$ in different clones (data not shown), probably reflecting differences in the number of pSUPER-HSF1 copies integrated and/or differences in the integration sites. At the end of selection, three clones presenting $>95\%$ reduction in HSF1 levels (HeLa-pSUPER-HSF1i clones 3, 16, and 31) were chosen for further characterization.

HeLa-pSUPER-p cells and HeLa-HSF1i clones were subjected to heat shock at 43°C for 1 hour, and whole-cell extracts were analyzed for HSF1 activation by EMSA and for HSF1 protein levels by Western blot. As shown in Fig. 2*A* (*top*), HSF1 activation was detected after heat shock in HeLa-pSUPER-p cells but not in HeLa-HSF1i clones. According to the gel retardation analysis results, both HSF1 protein (78 kDa) and its phosphorylated form (90 kDa, normally found after heat shock) were detected in HeLa-pSUPER-p cells but not in any of the HeLa-HSF1i clones (anti-HSF1; Fig. 2*A*). Differently from HSF1, levels of the HSF2, the p65 subunit of the

transcription factor nuclear factor- κB (NF- κB) and β -actin were similar in HeLa-pSUPER-p cells and HeLa-HSF1i clones, confirming a selective silencing of HSF1.

Interestingly, a reduction in HSF2 protein levels was observed in HeLa-HSF1i clones after heat shock. It has been reported that, during heat shock, HSF2 undergoes reversible inactivation associated with the conversion from a soluble to an aggregated form (24). The lower levels of HSF2 observed in HeLa-HSF1i clones suggest the possibility that HSF2 may be more thermolabile in the absence of HSF1 and/or in conditions of a defective HSR.

In parallel experiments, HeLa-pSUPER-p cells and HeLa-HSF1i clones growing on coverslips were subjected to heat shock at 43°C for 1 hour and analyzed for heat shock-dependent changes in HSF1 intracellular localization by confocal microscopy using anti-HSF1 antibodies. As shown in Fig. 2*B*, on exposure to heat shock, HSF1 relocalizes to form brightly stained nuclear foci in HeLa-pSUPER-p cells (25). HSF1 granules were not detected in HeLa-pSUPER-HSF1i clones (Fig. 2*B*, *left*).

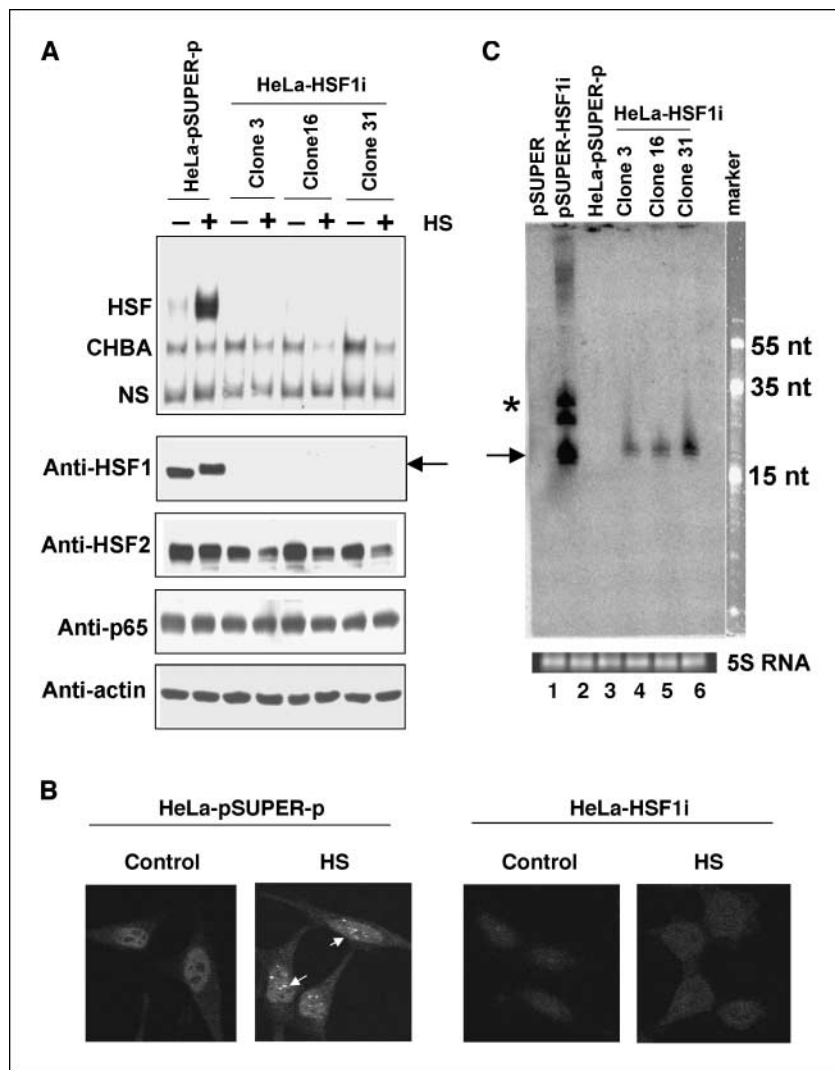
Finally, the expression of HSF1-specific siRNA was determined in all clones. Total RNAs extracted from HeLa-pSUPER-p, HeLa-HSF1i, or HeLa cells transiently transfected with the empty pSUPER or pSUPER-HSF1i vectors were analyzed for HSF1-specific siRNA expression by Northern blot. Blots were probed with a ^{32}P -labeled sense 19-nucleotide HSF1 probe corresponding to the targeting sequence and visualized by PhosphorImager. As shown in Fig. 2*C*, in HeLa cells transiently transfected with the pSUPER-HSF1i vector, both the stem-loop precursors and the siRNAs were detected. In all HeLa-HSF1i clones tested, only the HSF1-specific siRNA was visualized. HSF1-siRNA was still detected in HeLa-HSF1i clones after 6 months in culture.

siRNA-mediated HSF1 silencing is not affected by hyperthermic treatment. As indicated above, knockdown of HSF1 function did not cause major alterations in HeLa cell proliferation and morphology under normal growth conditions. In addition, no significant differences in cell sensitivity to mild heat shock conditions (up to 43°C for 1 hour) were detected (data not shown). The temperature of 43°C for 1 hour was then selected as a standard hyperthermic treatment condition. Because little is

known on the effect of temperature on siRNA-mediated gene silencing in mammalian cells, we next examined the effect of hyperthermic treatment on HSF1 silencing in HeLa-HSF1i clones. HeLa-pSUPER-p and HeLa-HSF1i cells were either kept at 37°C or subjected to hyperthermic treatment and then allowed to recover at 37°C. At different times during the recovery period, whole-cell extracts were analyzed for HSF1 DNA-binding activity by EMSA and for HSF1, HSP90, HSP70, and HSP27 protein levels by Western blot. As expected, in HeLa-pSUPER-p cells, HSF1 activation was detected at the end of the heat shock period and continued for 1.5 hours at 37°C, after which time it declined rapidly (Fig. 3, top). The slow-migrating phosphorylated HSF1 form was also detected at the time of HSF activation. HSF1 activation was rapidly followed by sustained HSP90, HSP70, and HSP27 synthesis (Fig. 3). Differently from HeLa-pSUPER-p cells, neither HSF1 activation nor HSP synthesis was detected at any time in HeLa-HSF1i clones (Fig. 3). Lack of HSP synthesis in heat-shocked HeLa-HSF1i cells was also confirmed in a parallel experiment by SDS-PAGE analysis of proteins after [³⁵S]methionine labeling (data not shown). Finally, the fact that the HSF1 protein was not detected over prolonged periods (up to 24 hours) following hyperthermic treatment (data not shown) indicates that siRNA-mediated HSF1 silencing is not repressed by heat shock.

HSF1 gene silencing does not alter HeLa cell sensitivity to cisplatin. To determine the effect of HSF1 silencing on cancer cell sensitivity to chemotherapy, HeLa-HSF1i clones and HeLa-pSUPER-p cells were plated (3×10^5 per well) in culture medium containing 5% FCS and treated with different concentrations of cisplatin. After 24 hours, cytotoxicity and the percentage of apoptotic cells were determined by MTT assay and DAPI staining, respectively. In both types of cells, cisplatin at concentrations of 40 to 100 μmol/L induced massive (~100%) apoptosis, whereas it had no effect at the concentration of 10 μmol/L (Fig. 4A). Lack of toxicity of cisplatin at the concentration of 10 μmol/L was confirmed in both types of cells by MTT assay (data not shown). In parallel experiments, the effect of 10 μmol/L cisplatin on cell cycle progression was determined by fluorescence-activated cell sorting (FACS) analysis of cell cycle phase distribution after propidium iodide staining at 24 and 48 hours after treatment. As shown in Fig. 4B (top), HeLa-pSUPER-p cells and HeLa-HSF1i clones showed identical cell cycle phase distribution profiles, with 62.3% of cells being in G₀-G₁, 8.4% in G₂-M, and 29.3% in S phase. At 24 hours after cisplatin treatment, both types of cells accumulated in S phase (~89%), whereas 11.3% cells were found in G₂-M and no cells were detected in G₀-G₁. At 48 hours after treatment, ~90% of cells accumulated in G₂-M, whereas 9.8% were found in

Figure 2. Characterization of HeLa clones with stable suppression of HSF1. **A**, HeLa-pSUPER-p cells and HeLa-HSF1i stable clones 3, 16, and 31 were subjected to heat shock at 43°C for 1 hour (+HS) or kept at 37°C (-HS), and whole-cell extracts were analyzed for HSF activation by EMSA. *Top*, positions of the HSF DNA-binding complex (HSF), constitutive HSE-binding activity (CHBA), and nonspecific protein-DNA interaction (NS); *bottom*, equal amounts of protein from the same samples were analyzed by Western blot for HSF1 (anti-HSF1), HSF2 (anti-HSF2), NF-κB p65 subunit (anti-p65), and β-actin. *Arrow*, position of the low-mobility phosphorylated HSF1 isoform. EMSA and Western blot analysis of one representative experiment of three with identical results. **B**, HeLa-pSUPER-p cells and HeLa-HSF1i clones growing on coverslips were either subjected to heat shock at 43° for 1 hour (HS) or kept at 37°C (control) and then analyzed for heat shock-dependent changes in HSF1 intracellular localization by immunofluorescence. *Arrows*, HSF1 stress granules. Results from HeLa-HSF1i clone 3. The same results were obtained from HeLa-HSF1i clones 16 and 31 (data not shown). **C**, HeLa cells transiently transfected with empty pSUPER or pSUPER-HSF1i vectors (lanes 1-2), HeLa-pSUPER-p cells (lane 3), and HeLa-HSF1i clones 3, 16, and 31 (lanes 4-6) were analyzed for HSF-specific siRNA expression by Northern blot using a ³²P-labeled 19-nucleotide sense HSF1 probe corresponding to the targeting sequence. *Top*, *asterisk*, stem-loop precursors; *arrow*, siRNAs. 5'-Fluorescein-labeled 55-nucleotide (55 nt), 35-nucleotide (35 nt), and 15-nucleotide (15 nt) oligos were used as markers. *Bottom*, control 5S rRNA band detected by ethidium bromide staining.



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the S phase and no cells were detected in G₀-G₁ in both types of populations (Fig. 4B, bottom). These results indicate that HSF1 silencing did not alter the response of HeLa cells to low-dose cisplatin treatment. The 10- μ mol/L concentration of cisplatin was then selected for further studies.

HSF1 gene silencing sensitizes HeLa cells to combined hyperthermia/cisplatin treatment. We next analyzed the effect of HSF1 silencing in cells exposed to combined hyperthermia/cisplatin treatment under conditions in which each treatment was ineffective in inducing apoptosis in both HeLa-pSUPER-p and HeLa-HSF1i cells when administered separately. HeLa-HSF1i clones and HeLa-pSUPER-p cells were treated with 10 μ mol/L cisplatin and, after 2 hours, subjected to hyperthermic treatment. After 24 hours, cells were analyzed by Western blot for PARP cleavage as a biochemical marker of apoptosis, for HSP70 level as an indicator of HSR activation, and for β -actin as loading control. In a parallel experiment, the percentage of apoptotic cells was determined by microscopic evaluation of nuclear morphology after DAPI staining.

Consistent with the results described in Figs. 3 and 4, no significant differences in apoptosis evaluated both by PARP cleavage analysis (Fig. 5A) and by DAPI staining (Fig. 5B) were

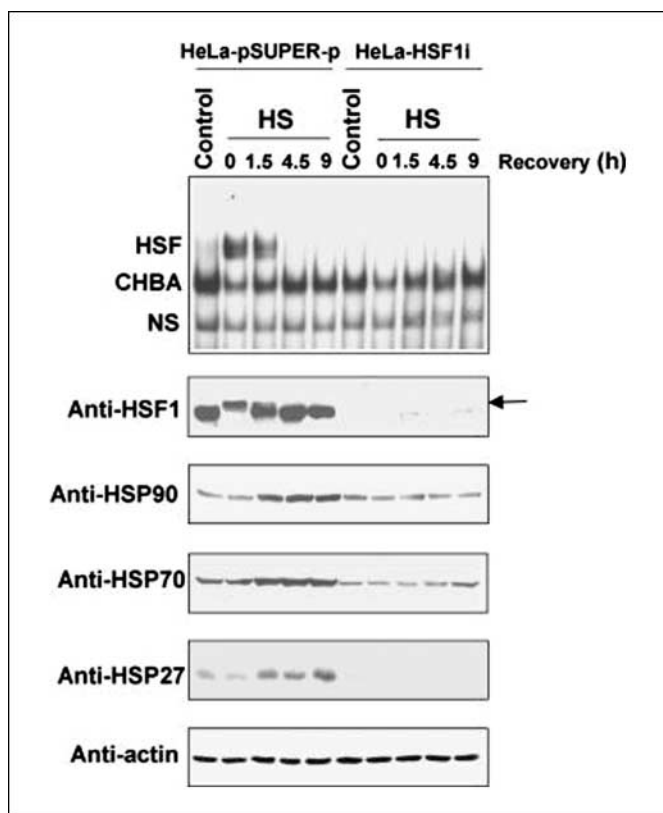


Figure 3. RNAi-induced HSF1 stable suppression is not affected by heat shock. HeLa-pSUPER-p cells and HeLa-HSF1i clone 3 were either kept at 37°C (control) or subjected to heat shock at 43°C for 1 hour and allowed to recover at 37°C. After 1 hour at 43°C (time 0) or at 1.5, 4.5, and 9 hours during the recovery period at 37°C, whole-cell extracts were analyzed for HSF DNA-binding activity by EMSA (top) and for HSF1 (anti-HSF1), HSP90 (anti-HSP90), HSP70 (anti-HSP70), HSP27 (anti-HSP27), and β -actin (anti-actin) levels by Western blot (bottom). Arrow, position of the low-mobility phosphorylated HSF1 isoform. Similar results were obtained with HeLa-HSF1i clones 16 and 31. EMSA and Western blot analysis of one representative experiment of three with identical results.

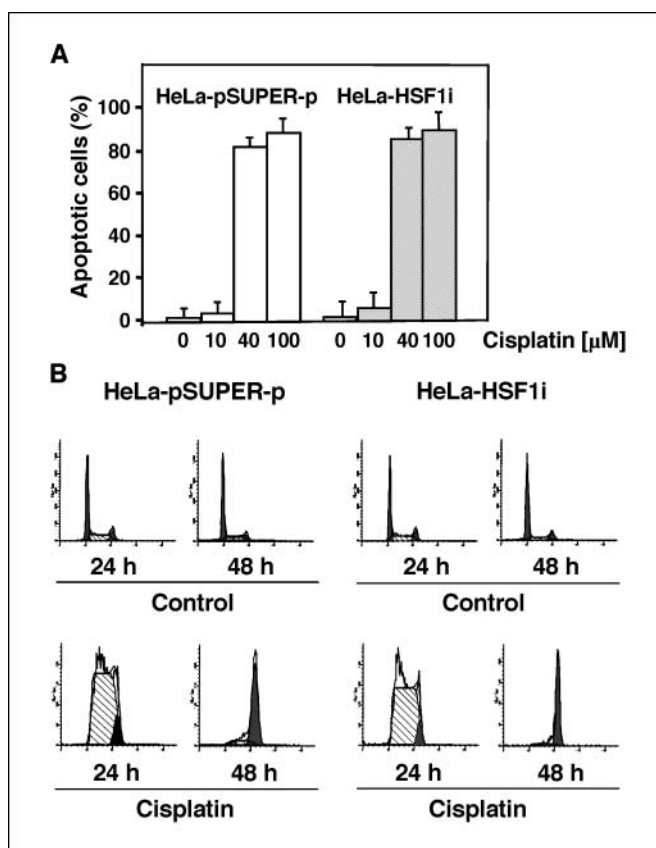


Figure 4. Stable HSF1 gene suppression does not affect HeLa cell response to cisplatin. A, HeLa-pSUPER-p (empty columns) and HeLa-HSF1i clone 3 (dashed columns) cells treated with different doses of cisplatin or control diluent for 24 hours were analyzed by microscopy after DAPI staining to determine the percentage of apoptotic cells. Columns, mean of three independent experiments; bars, SD. B, HeLa-pSUPER-p (right) and HeLa-HSF1i clone 3 (left) cells were treated with 10 μ mol/L cisplatin (bottom) or control diluent (top) and, after 24 and 48 hours, analyzed by FACS. Profiles of DNA content measurement from one of three experiments with identical results. Similar results were obtained with HeLa-HSF1i clones 16 and 31.

observed in HeLa-HSF1i clones compared with HeLa-pSUPER-p cells when hyperthermia and cisplatin treatments were administered separately.

Interestingly, opposite results were detected when cells were exposed to combined hyperthermia/cisplatin treatment. In fact, under these conditions, whereas HeLa-pSUPER-p cells did not respond, ~95% of HeLa-HSF1i cells underwent apoptosis and died at 24 hours after treatment (Fig. 5A and B). To exclude the possibility that HeLa-HSF1i cell sensitivity to apoptosis was due to clonal differences, HeLa-HSF1i-p cells were treated identically and analyzed as described for HeLa-HSF1i clones. Although less pronounced, similar effects were detected in HeLa-HSF1i-p cells: induction of apoptosis was observed in >50% HeLa-HSF1i-p cells after combined hyperthermia/cisplatin treatment, accordingly to the fluctuation in HSF1 suppression observed during the cloning procedure (data not shown). Similar effects (~40-50% apoptosis in two separate experiments) were also detected in human colon carcinoma HCT-116 cell pools stably transfected with the pSUPER-HSF1i vector.³

³ A. Rossi et al., preliminary results.

Coexpression of the major HSP90, HSP70, and HSP27 HSPs partially reverts sensitivity to hyperthermia/cisplatin-induced apoptosis in HeLa-HSF1i clones. To verify whether the acquired sensitivity to hyperthermia/cisplatin-induced apoptosis observed in HeLa-HSF1i clones was due to an impairment in HSP expression, HeLa-HSF1 cells were transfected with the empty pcDNA3 vector or with pcDNA3 vectors expressing the Flag-tagged forms of HSP90, HSP70, and HSP27 either individually or in combination. At 16 hours after transfection, cells were treated with 10 $\mu\text{mol/L}$ cisplatin and, after 2 hours, subjected to hyperthermic treatment. At 24 hours after treatment, cells were analyzed for PARP cleavage and for levels of Flag-tagged HSP90, HSP70, and HSP27 and β -actin by Western blot. Levels of PARP cleavage were quantified by VersaDoc 1000 using Quantity One software. As expected, hyperthermia/cisplatin cotreatment resulted in complete PARP cleavage (Fig. 6A). As shown in Fig. 6B, although a slight reversion in PARP cleavage was observed when HeLa-HSF1i cells were transfected with HSP27, HSP70, and HSP90 separately (10% for HSP27 and HSP90 and 18% for HSP70), a relevant effect was obtained only when all three HSPs were coexpressed (50% reduction in PARP cleavage compared with HeLa-HSF1i cells transfected with the empty vector; Fig. 6A and B). Considering that the transfection efficiency of these cells was $\sim 70\%$, this result seems to be greatly significant.

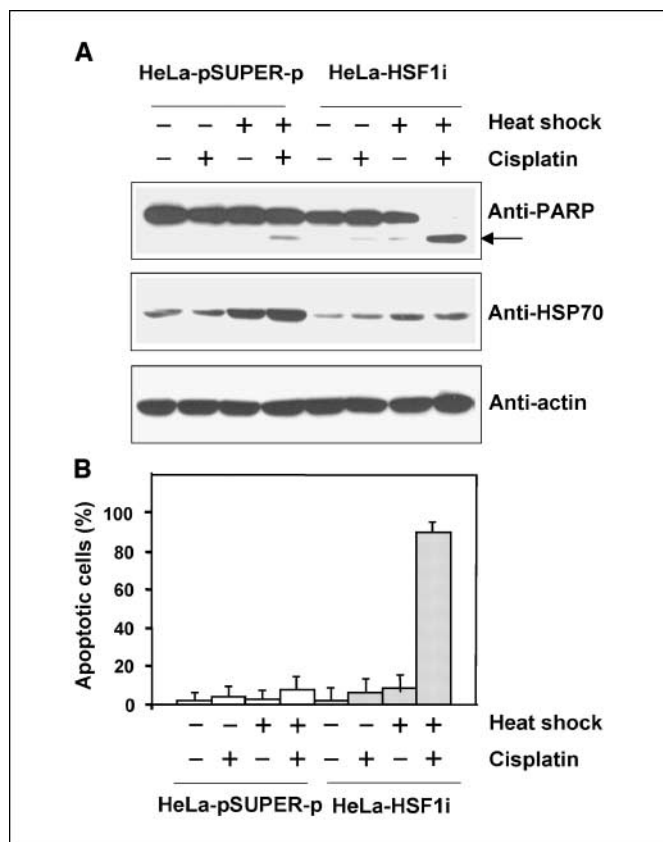


Figure 5. HSF1 gene suppression sensitizes HeLa cells to hyperthermia/cisplatin-induced apoptosis. A, HeLa-pSUPER-p and HeLa-HSF1i clone 3 cells were treated with 10 $\mu\text{mol/L}$ cisplatin or control diluent for 2 hours and then subjected to heat shock for 1 hour at 43°C. After 24 hours, levels of PARP (top), HSP70 (middle), and β -actin (bottom) were determined by Western blot analysis. Arrow, 85-kDa PARP apoptotic fragment. B, in parallel samples, the percentage of apoptotic cells was determined by DAPI staining. Columns, mean of three independent experiments; bars, SD. Similar results were obtained with HeLa-HSF1i clones 16 and 31.

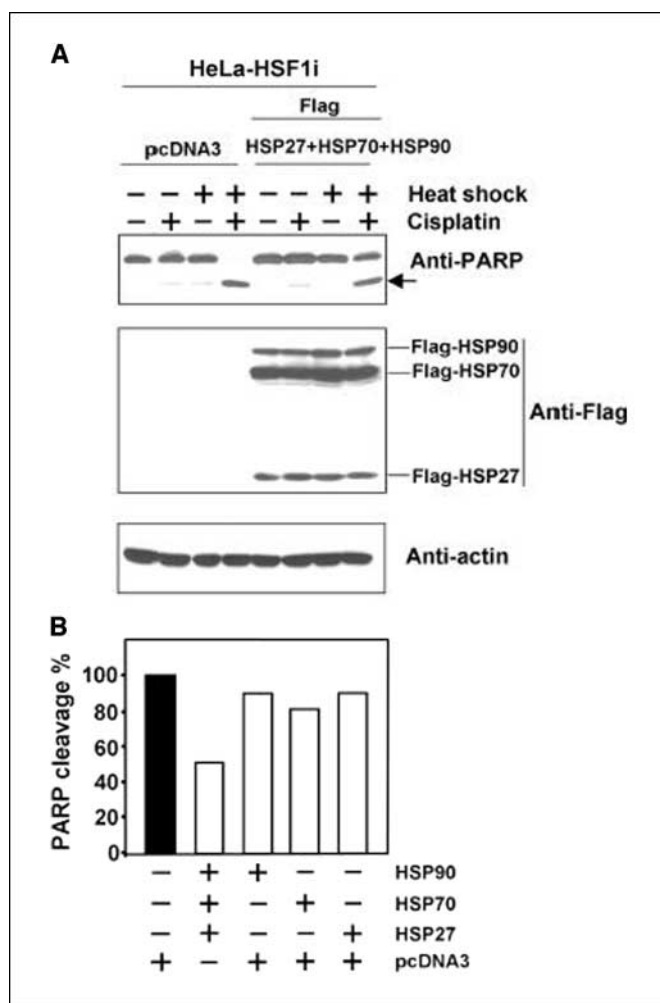


Figure 6. Coexpression of HSP90, HSP70, and HSP27 partially restores resistance to hyperthermia/cisplatin treatment. A, HeLa-HSF1i clone 3 cells were transfected with the empty pcDNA3 vector or cotransfected with pcDNA3 vectors expressing Flag-tagged HSP27, HSP70, and HSP90 proteins. After 16 hours, transfected cells were treated with 10 $\mu\text{mol/L}$ cisplatin (+) or control diluent (-). Two hours after cisplatin treatment, cells were subjected to heat shock for 1 hour at 43°C (+) or kept at 37°C (-). After 24 hours at 37°C, cells were analyzed by Western blot with antibodies to PARP (top), Flag (middle), and β -actin (bottom). Arrow, 85-kDa PARP apoptotic fragment. Similar results were obtained with HeLa-HSF1i clones 16 and 31. B, in a parallel experiment, HeLa-HSF1i clone 3 cells were transfected with the empty pcDNA3 vector or cotransfected with pcDNA3 vectors expressing Flag-tagged HSP27, HSP70, and HSP90 proteins either individually or in combination and treated as described in (A). After 24 hours at 37°C, cells were analyzed by Western blot using anti-PARP antibodies and PARP cleavage was quantified by VersaDoc 1000 using Quantity One software. Data from samples cotreated with cisplatin and heat shock. Each experiment was repeated thrice with the same results.

Discussion

Carcinoma of the uterine cervix is the second most common female cancer worldwide (26). Improvements in the early detection of cervical cancers have substantially reduced the associated mortality rates over the past decades; however, in patients diagnosed with advanced cervical cancers, the mortality rates have remained unchanged over the past 25 years (27, 28). Currently, advanced cervical cancer is treated with radiotherapy and cisplatin-based chemotherapy; nevertheless, unfortunately, most patients will eventually relapse, and improved treatment options for this type of malignancy are highly needed.

One of the modalities that has been applied in the clinical setting to enhance the efficacy of cisplatin chemotherapy in cervical carcinoma is hyperthermia. In combination with hyperthermia, cisplatin cytotoxicity has been shown to increase almost linearly with the increase in temperature (13, 29). The exact mechanism of potentiation remains to be elucidated. In addition to an increase in blood flow and oxygenation in the tumor, an increase in intracellular cisplatin uptake, as well as increased DNA damage and impairment of DNA strand break repair, has been implied in the synergistic effect (29). However, as indicated above, the efficacy of hyperthermochemotherapy may be dampened by the cytoprotective effect of HSPs induced by hyperthermic treatment. Consequently, the block of HSP expression would be expected to enhance cancer cell sensitivity to hyperthermochemotherapy. HSF1, being the main regulator of the entire repertoire of stress-inducible HSPs, may then represent a critical target for anticancer therapy. To verify this hypothesis, in the present study, we aimed at targeting HSF1 by siRNA to disrupt hyperthermia-induced cytoprotective effects and to selectively amplify damages in cancer cells.

Our first goal was to create a vector expressing a small hairpin RNA able to potently suppress HSF1 expression. A pSUPER-HSF1i was successfully produced and used to obtain a cervical carcinoma cell line (HeLa-HSF1i) with stable loss of HSF1 function. Differently from control HeLa-pSUPER-p cells that present high HSF1 protein levels, in HeLa-HSF1i clones, HSF1 protein was undetectable either under normal growth conditions or after heat shock. In addition, neither HSF1 DNA-binding activity nor HSP (HSP90, HSP70, and HSP27) induction was detected in HeLa-HSF1i cells under different types of stress conditions, including heat shock up to 45°C and exposure to the HSP90 inhibitor geldanamycin (30) or to HSP inducers, such as different cyclopentenone prostanoids (data not shown; ref. 31). Furthermore, the suppression was selective for the *HSF1* gene because intracellular levels of the related HSF2 were unaffected.

An interesting aspect to take into consideration during the generation of a HSF1 knockdown population is the long-term effect of temperature on RNAi. A temperature-dependent gene silencing by RNAi has been reported in *Drosophila* (32). In this case, the RNAi effect on *Drosophila* sex differentiation observed at 29°C was strongly inhibited at 22°C. A temperature-sensitive RNAi effect was also reported as an antiviral reaction in plant systems (33). In mammalian cells, although RNAi suppression has been reported during hypothermic treatment in transient transfection experiments (34), nothing is known about the effects of heat shock on RNAi. The fact that HSF1 was not detected up to 24 hours after a 43°C heat shock in HeLa-HSF1i clones shows for the first time that siRNA interference is not reversed or inhibited by heat, which is an important observation in view of possible clinical applications. However, it remains to be established whether this represents a general phenomenon or is restricted to HSF1.

The siRNA-mediated HSF1 knockdown in HeLa-HSF1i clones is maintained over long periods (>6 months), and its transcript product is not toxic to HeLa cells because no selection against HSF1 knockdown was observed. In addition, no major differences in cell growth or cell cycle distribution were detected in HeLa-HSF1i clones compared with control cells. Interestingly, no differences in cell survival between HSF1 knockdown and control cells were detected also after exposure to heat shock up to 43°C for 1 hour. The observed resistance of HeLa-HSF1i cells to a 43°C heat shock-induced damage could reflect the fact that HeLa cells, as several other human cancer cell lines, express constitutively elevated levels of HSP70 (35). The molecular mechanism respon-

sible for HSP70 overexpression in cancer cells is not known, but HSF1-independent mechanisms have been described (36, 37). The hypothesis of a HSF1-independent constitutive expression of HSP70 in HeLa cells is further supported by the fact that, in our model, only a slight reduction in constitutive HSP70 levels was observed in HSF1 knockdown clones. In addition, it cannot be excluded that, although HSF1 is the primary regulator of the stress response in normal eukaryotic cells, other stress factors may come into play in cancer cells. Whereas the "buffer capacity" of constitutive HSP70 was able to counteract the damaging effects of heat at 43°C, HSF1 knockdown cells were instead found to be much more sensitive to higher temperatures (45°C for 1 hour) than the control HeLa-pSUPER-p population (data not shown).

No major differences in the response to cisplatin were found between HeLa-HSF1i and HeLa-pSUPER-p cells under the conditions examined. In fact, both types of cells underwent massive apoptosis after a 24-hour treatment with cisplatin at concentrations >40 µmol/L, whereas a similar alteration in cell cycle distribution, resulting in a G₂ phase block at 48 hours after treatment, was observed at 10 µmol/L cisplatin. These results are in accord with previous work by Bagatell et al. (30), who found no differences between HSF1 knockout cells and wild-type fibroblasts in response to cisplatin or doxorubicin treatment.

Surprisingly, a completely different scenario was detected after combined hyperthermia/cisplatin treatment. In fact, whereas HeLa-pSUPER-p cells did not respond to hyperthermochemotherapy, ~95% of HeLa-HSF1i cells underwent apoptosis after 24 hours of treatment, suggesting that HSP synthesis could be involved in cytoprotection under these conditions.

HSPs have an extremely complex role in the regulation of apoptosis. In particular, HSP70, HSP27, and HSP90 have been shown to inhibit apoptosis by direct physical interaction with apoptotic molecules (16, 17). Because of their critical role in the control of apoptosis, different strategies targeting the function or the expression of these HSPs have been proposed in cancer treatment (10, 17, 38). To investigate the role of the major HSP in cytoprotection during hyperthermochemotherapy in our system, we have expressed HSP90, HSP70, and HSP27 proteins either individually or in combination in HeLa-HSF1i cells by transient transfection. The results indicate that the coordinate expression of different HSP is necessary to obtain optimal antiapoptotic effects after hyperthermia/cisplatin treatment, suggesting that inhibition of the HSR via HSF1 could result in a more efficient strategy to enhance hyperthermochemotherapy than targeting HSPs singularly.

As indicated above, improved treatment options for invasive cervical cancer are highly needed. The advanced capability to deploy heat with more sophisticated modalities has led to a resurgence in interest in hyperthermia for cervical cancer especially in combination with either radiation or cisplatin (13, 39). Recently, the combination of radiotherapy with hyperthermochemotherapy in a triple-modality treatment for advanced cervical cancer has been shown to increase both response rate and survival time in a phase II study and is currently in phase III trial (40, 41). The results described herein show that a further improvement in sensitivity to hyperthermochemotherapy may be obtained by targeting HSF1. We in fact show that the block of HSF1 function leads to massive apoptosis of cervical carcinoma cells exposed to hyperthermia in combination with cytostatic doses of cisplatin. It should be pointed out that, although the experiments described are limited to the study of hyperthermia in combination with cisplatin, we have found that HSF1 silencing also results in increased sensitivity of

HeLa cells to treatment with the HSP90 inhibitor geldanamycin and the I κ B kinase inhibitor 15-deoxy- $\Delta^{12,14}$ -prostaglandin J $_2$.⁴

All together, these findings identify HSF1 as a target for anticancer therapy, suggesting novel strategies based on RNAi approaches (42, 43) or on the development of selective HSF1 inhibitors to enhance therapeutic efficacy in cancer treatment.

⁴ A. Rossi and M.G. Santoro, unpublished observation.

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