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***Hsp27* antisense oligonucleotides sensitize the microtubular cytoskeleton of Chinese hamster ovary cells grown at low pH to 42°C-induced reorganization**

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Running Title: Antisense *hsp27* oligonucleotides sensitize CHO cells grown at low pH to heat.

Keywords: Hsp27, heat shock, antisense, CHO, microtubule cytoskeleton.

Abstract

Chinese hamster ovary (CHO) cells maintained *in vitro* at pH 6.7 were used to model cells in the acidic environment of tumors. CHO cells grown at pH 6.7 develop thermotolerance during 42°C heating at pH 6.7, and their cytoskeletal systems are resistant to 42°C-induced perinuclear collapse. Hsp27 levels are elevated in cells grown at pH 6.7 and are further induced during 42°C heating while Hsp70 levels remain low or undetectable, suggesting that Hsp27 is responsible for some of the novel characteristics of these cells. An antisense oligonucleotide strategy was used to test the importance of Hsp27 by lowering heat-induced levels of the protein. The response of the microtubular cytoskeleton to heat was used as an endpoint to assess the effectiveness of the antisense strategy. Treatment with antisense oligonucleotides prevented the heat-induced increase of Hsp27 levels measured immediately following heat. Treatment with antisense oligonucleotides also sensitized the cytoskeleton of cells grown at low pH to heat-induced perinuclear collapse. However, cytoskeletal collapse was not evident in cells grown at pH 6.7 and treated with 4-nt mismatch oligonucleotides or in control cells maintained and heated at pH 6.7. The cytoskeleton collapsed around the nucleus in cells cultured and heated at pH 7.3. These results confirm that overexpression of Hsp27 confers heat protection to the microtubular cytoskeleton in CHO cells grown at low pH.

1. Introduction

Mammalian cells cultured at pH 6.7 in vitro are being used to model the chronic acidic environment of tumor cells. We (1-3) and others (4-6) have shown that growth of rodent cells at low pH results in an altered phenotype. CHO cells grown at pH 6.7 are not sensitized to 42°C hyperthermia at pH 6.7 (3), presumably because their intracellular pH is similar to cells grown at pH 7.3 (1,3,7). In addition, CHO cells cultured at pH 6.7 have the ability to resist both the accumulation of nuclear associated proteins (NAPs) and disruption of the microtubular (8-9) and actin-microfilament (9) cytoskeletal systems during heating at pH 6.7. The endogenous Hsp27 levels are elevated in CHO cells grown at low pH (10), and Hsp27 levels are further increased during heating at 42°C at pH 6.7 (9). Hsp70 is not detectable at growth temperatures nor is it induced upon heating at pH 6.7, leading to our proposal that Hsp27 plays a dominant role in the conferral of the heat resistant, low pH phenotype to CHO cells grown at pH 6.7 (9). The possibility still remains that other heat shock proteins not examined may contribute to the heat resistant phenotype of cells cultured at pH 6.7.

Heat shock proteins are a set of conserved proteins found in all organisms, and they have been classified to families according to their relative molecular weights (11). Members of each family may show constitutive or inducible expression as well as be targeted to different compartments (12). Many heat shock proteins serve as molecular chaperones that recognize and bind to nascent polypeptides or unfolded proteins preventing irreversible protein aggregation that can lead to cell death (12). Heat shock proteins also represent a class of anti-apoptotic proteins that interact with apoptotic signaling cascades at multiple points blocking the cascades (13-16). Accordingly, heat shock proteins are often referred to as "survival proteins".

Exposure of a variety of cell types in vitro to 42-45°C have been shown to disrupt one or more cytoskeletal systems (17-19). The cytoskeletal systems affected vary with treatment temperature and cell type. All three cytoskeletal systems in CHO cells, namely the actin microfilament system, the microtubular system, and the vimentin intermediate filament system, undergo reorganization upon heat treatment (20-21).

Hsp27 may be responsible for maintenance of the cytoskeletal organization in heated, low pH adapted cells. Hsp27 is widely reported to chaperone the actin containing microfilament system. Transfected cells overexpressing Hsp27 are not only resistant to heat killing (22-24), but their F-actin cytoskeleton is stabilized against disruption by heat (23,25). Hsp27 exists as a monomer and as multimers (26). Unphosphorylated Hsp27 is an actin capping protein that prevents assembly of F-actin (27). Heat shock leads to the rapid phosphorylation of Hsp27 (28). Phosphorylation of Hsp27 leads to a loss of its ability to inhibit actin-polymerization and also dissociates the large multimers into smaller oligomers (29). Both stabilization of the actin CSK (prevention of disassembly) and resistance to heat killing are associated with the phosphorylation of Hsp27 and reduction of the multimer size of Hsp27 to smaller oligomers (25, 30-31). Lavoie *et al.* (23), using clones of transfected cells, demonstrated that Hsp27 not only needed to be elevated prior to heat shock in order to confer resistance to heat killing, but that the Hsp27 had to be phosphorylated in order to confer resistance to the actin cytoskeleton (25, 30, 32). However, studies with a chimera protein consisting of enhanced green fluorescence protein fused to the N-terminus of human Hsp27 indicated that reduction in oligomer size was not a requirement for protection against heat-induced cell killing (33). Therefore, nonphosphorylated HSP27 can protect as a molecular chaperone, and phosphorylated HSP27 can protect the actin cytoskeleton from stress, and both forms of protection enhance cell survival.

Heat shocks between 42.5° and 50°C have been shown to inactivate microtubular proteins and disassemble microtubules *in vitro* (34), with the degree of inactivation greater for the higher temperatures. The ability of a 43°C heat treatment to induce reorganization of the microtubular cytoskeleton was partially abrogated in thermotolerant CHO cells (35). These cells contained elevated levels of several heat shock proteins including Hsp27. Hsp27 has been shown to associate with microtubules in HeLa cells (36), and alpha B-crystallin forms a complex with tubulin dimers from L6 myoblasts (37). Furthermore, the small heat shock/alpha-crystallin protein, p26, from encysted *Artemia* embryos associates with tubulin isolated from the embryos and suppresses its denaturation by heat (38). Recently alpha B-crystallin was shown to protect against denaturation of tubulin derived from L6 myoblast lysates and to bind to microtubule associated proteins, protecting microtubules in myoblast and glioma cells from stress induced disassembly (39). Therefore, Hsp27 acts as a chaperone to protect tubulin from denaturation and protects intact microtubules from heat-induced disassembly. The importance of the phosphorylated form of Hsp27 for protection of the microtubular cytoskeleton from thermal and other stresses is not known.

The contribution of Hsp27 to the phenotype of CHO cells cultured and heated at pH 6.7 was tested using antisense *hsp27* oligonucleotides. The stability of the microtubular cytoskeleton, an endpoint amenable to the use of antisense oligonucleotides, was related to Hsp27 expression. Antisense oligonucleotide strategies have been used to inhibit expression of target genes in a wide variety of biological systems to confirm a functional relationship (40-43). Antisense strategies have shown sequence specificity and isotype specificity in cells (40,44), animals (45-46) and humans (47). An antisense oligonucleotide strategy has been successfully used to confirm that resistance to doxorubicin is attributable to high levels of Hsp27 in MCF-7

cells (48). Here we tested the ability of antisense *hsp27* oligonucleotides to inhibit the 42°C-induction of Hsp27 in order to document the relationship between high levels of Hsp27 and the stability of the microtubular cytoskeleton in CHO cells cultured at pH 6.7.

2. Materials and Methods

2.1. Cell Culture

CHO cells were maintained at either pH 7.3 or pH 6.7 and frozen stocks prepared. Cells were grown at pH 6.7 for 90 days prior to freezing. Frozen stocks were thawed and cultured at pH 6.7 or pH 7.3, respectively, for one week prior to any experiment. All cell cultures were maintained in McCoy's 5A media (modified) (GibcoBRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum and antibiotics (0.07 mg/ml penicillin; 0.1 mg/ml streptomycin). The pH of the growth medium was adjusted to pH 6.7 by reducing sodium bicarbonate in the medium from 2.2 g/l to 0.466 g/l. The concentration of NaCl was adjusted to maintain osmolarity.

2.2. Oligonucleotides

Oligodeoxynucleotides representing antisense and 4-nt mismatch sequences of hamster *hsp27* were obtained from Annovis (Aston, PA). Antisense and 4-nt mismatch oligonucleotides containing phosphorothioate linkages were as follows: antisense oligonucleotide (bp 31 to 17) (5'-CTCGGTCATGTTCTT-3') and 4-nt mismatch oligonucleotide (bp 31 to 17) (5'-CTCGCTGAAGATCTT-3').

2.3. *Cell Treatment*

CHO cells cultured at pH 7.3 and pH 6.7 were plated (5×10^4 cells) into 60 mm tissue culture dishes. The pH 6.7 medium was replaced after one hour with pH 7.3 medium containing 4 μ M oligonucleotides (antisense or mismatch) and calf thymus histones (4.8 μ g/ml) (Sigma, St. Louis, MO) of equal weight proportions to form electro-neutral multimolecular complexes that enhance cellular uptake of oligonucleotides. Additional oligonucleotides (4 μ M) and histones (4.8 μ g/ml) were added each day throughout the experiment. Medium was replenished at 48 hours and fresh oligonucleotides and histones added. All cells were trypsinized after 96 hours and plated either onto dry fibronectin-coated 22 x 22 mm coverslips (2.5×10^4 cells) (Biocoat, Becton-Dickson, Bedford, MA) that had been placed into 60 mm Petri tissue culture dishes or into 60 mm Petri tissue culture dishes containing five ml of medium at pH 7.3 or pH 6.7. Equilibrated medium (5 ml) was added after 15 minutes to the dishes containing the coverslips and the dishes were maintained for two additional hours at 37°C in the CO₂ incubator. The tissue culture dishes were sealed with PetriSEAL (DiversifiedBiotech, Boston, MA) to provide a waterproof and gas proof seal just prior to heating by submersion in 42°C waterbaths. The cells ~~on coverslips and tissue culture dishes~~ were heated at 42°C (4 h) starting 2 - 2.5 h after plating. The cells in 60 mm dishes were processed for western blot analysis immediately after heating. The cells on coverslips were processed for cytoskeletal organization by staining for tubulin immediately after heating.

2.4. *Western blot analysis*

Treated cells were washed twice in PBS (5 ml) and 0.5 ml of lysis buffer (62.5 mM Tris-HCl, pH 6.8, 1% glycerol, 0.5% SDS, 1 mM EDTA, 40 mM DTT, 5 mM 4-(2-aminoethyl)-

benzenesulfonyl fluoride, 0.7 mg/L pepstatin, and 14 mg/L aprotonin) was added to each dish. The cells were scraped with a plastic policeman and transferred to a 1.5 ml microfuge tube. The lysate was boiled for seven minutes and then sonicated for 10 seconds. Protein determinations were carried out using the Bradford assay (49). Equivalent amounts of total protein (8 µg) were loaded onto the lanes of 12% polyacrylamide gels containing 0.1% SDS and electrophoresed. Proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane using a semi-dry transfer apparatus (Pharmacia-LKB Multi-Phor II) containing transfer buffer (48 mM Tris, 39 mM glycine, 20% methanol, and 0.0376% SDS). The membrane was processed for antibody detection using a Tropix Western-Star protein detection kit (Applied Biosystems, Foster City, CA). The membrane was incubated in blocking buffer containing PBS, 0.1% I-Block (supplied in kit) for one hour at room temperature or overnight at 4°C. Rabbit anti-Hsp27 polyclonal antibody (1:500) (L2R3, a gift from Dr. Jacques Landry) and mouse anti-GAPDH monoclonal antibody (1:100 000) (Chemicon, Temecula, CA) in blocking buffer were added and incubated for one hour. The membranes were washed three times five min each in blocking buffer. The membranes were then treated with secondary antibody [rabbit anti-IgG (1:20 000) and mouse anti-IgG (1:10 000) supplied in the kit] conjugated with alkaline phosphatase in blocking buffer for one hour. The membranes were washed three times five min each in blocking buffer and then twice in Assay buffer (supplied in the kit) for two min each. The blots were then placed between layers of plastic wrap containing three ml of Substrate solution (supplied in the kit). After five min the blots were exposed to Hyperfilm ECL (Amersham Pharmacia Biotech, Piscataway, NJ) and processed.

2.5. *Tubulin Staining*

Cells attached to coverslips were washed twice in Pipes CSK buffer (10 mM Pipes, pH 6.8, 100 mM KCl, 3.3 mM MgCl₂, 0.33 M sucrose, 1 mM EGTA) for five min each. The cells were permeabilized and fixed in ice-cold methanol for four min. After washing three times in PBS, the coverslips were blocked for 30 min to 60 min in PBS, 1% BSA. The coverslips were incubated with rat anti-alpha tubulin antibody (MCA P77, Serotec Ltd, Oxford, UK) at 1:100 dilution in PBS, 1% BSA for one hour and then washed three times in PBS, 1% BSA. The coverslips were incubated at room temperature with goat anti rat-IgG AlexaFluor-594 (1:1000) (Molecular Probes, Eugene, OR) for 30 minutes in the dark. After washing three times for five min each in PBS, the nuclei were stained with DAPI (0.6 μM DAPI in PBS) for five min. The coverslips were rinsed in deionized water and mounted in Gelvatol (Monsanto) mounting medium for viewing. Images from a Nikon Microphot FX microscope integrated with an intensified Hamamatsu camera were digitized, captured and analyzed using Metamorph software (Universal Imaging Corp. PA). Approximately 200-500 individual cells were analyzed for each endpoint utilizing two coverslips for each endpoint per experiment. Bar graphs were prepared showing the size distribution between heated and unheated cells.

2.6. *Statistics*

Student's t-test was used to determine the significance (p-values) of the cytoskeletal size distribution between heated and unheated cells.

3. Results

3.1. *Effects of antisense oligonucleotides on Hsp27 protein levels*

Cells were heated at 42°C for 4 h following 96 h of exposure to the antisense oligonucleotides or 4-nt mismatch oligonucleotides and Hsp27 expression monitored. The induction of Hsp27 by 42°C was prevented in cells treated with antisense oligonucleotides (figure 1, lanes 3 and 4). Cells grown at pH 7.3 (figure 1, lanes 7 and 8), cells grown at pH 6.7 (figure 1, lanes 5 and 6) or pH 6.7 cells maintained at pH 7.3 for four days (not shown) and not exposed to oligonucleotide treatment showed induction of Hsp27 during heating. Hsp27 was also induced during heating in cells treated with 4-nt mismatch oligonucleotides (figure 1, lanes 1 and 2). Surprisingly, the level of endogenous Hsp27 was not reduced in cells grown at pH 6.7 by the 4 day treatment with antisense oligonucleotides (lane 3). These results indicate that while the antisense strategy did not lower pre-existing endogenous levels of Hsp27, it did inhibit induction of Hsp27 and, therefore, limit the total amount of Hsp27 in the cell during heating.

The microtubular cytoskeleton of CHO cells cultured and heated at pH 6.7 is normally resistant to 42°C induced perinuclear collapse (8;9). The effect of antisense *hsp27* oligonucleotides on the cytoskeletal response was examined in cells grown at pH 6.7 and heated at pH 6.7 (figure 2). Cytoskeletal reorganization was clearly evident in cells treated with antisense oligonucleotides and heated (figure 2f), as well as in cells grown and heated at pH 7.3 (figure 2b). The cytoskeletal system of cells grown at pH 6.7 (figure 2c) is resistant to heat-induced cytoskeletal collapse (figure 2d).

Quantification of the effects of heat on the microtubular cytoskeleton of CHO cells is summarized in figure 3. The area of the cytoskeleton of cells grown at pH 7.3 and heated at pH 7.3 decreased significantly ($p < 0.001$) due to microtubule collapse (compare figure 3a and 3b).

Cytoskeletal collapse is indicated by a decrease in the mean area of the cytoskeleton in heated cells compared to the mean area of the cytoskeleton in unheated cells. The cytoskeleton does not collapse in cells maintained and heated at pH 6.7; rather, the area of the cytoskeleton increases during heating (compare figure 3d and 3c). However, CHO cells grown at pH 6.7 and treated with antisense oligonucleotides do exhibit cytoskeletal collapse ($p < 0.001$, compare figure 3g and 3h) upon heat treatment. The cytoskeleton of CHO cells grown at pH 6.7 and treated with the 4-nt mismatch oligonucleotides did not decrease in area during heat treatment, indicating that the antisense oligonucleotide effect was specific to the antisense oligonucleotides (compare figure 3i and 3j). Finally, cells grown at pH 6.7, maintained at pH 7.3 for 96 hours during the experiment, and heated at pH 6.7 did not exhibit cytoskeletal collapse (compare figure 3e and 3f). Hence, exposure of pH 6.7 grown cells to pH 7.3 for four days, namely, the conditions used for oligonucleotide uptake, had no effect on the cellular phenotype with respect to cytoskeletal stability.

4. Discussion

In the present study we show that *hsp27* antisense oligonucleotides inhibit the heat-induced expression of Hsp27 in cells grown at pH 6.7 and sensitize the microtubular cytoskeleton of these cells to heat-induced reorganization. These data confirm our previous proposal that Hsp27 plays a major role in conferring stability to the cytoskeleton in cells cultured and heated at pH 6.7 (9).

Treatment with the antisense oligonucleotides did not reduce the endogenous level of Hsp27 in the cells adapted to growth at pH 6.7. This unexpected finding was most likely due to a long half life of the protein. The induced protein has a half life of 13 h (28). It is unknown how

low pH_i induces elevated levels of Hsp27. It is assumed that the cells have increased transcription in response to the stress of being at a low pH_i. However, the half life of the protein may be increased further in cells adapted to growth at low pH. Thus one possible interpretation of the results is that the half life of the protein is increased in the cells adapted to growth at pH 6.7, and the 4 day period of incubation with the antisense oligonucleotides is not sufficient to deplete these cells of the Hsp27 protein.

Our data indicate that the stability of the microtubular cytoskeleton in cells cultured at pH 6.7 is due to the increased levels of Hsp27 found during heat shock rather than the elevated constitutive levels at 37°C. The heat-induced levels of Hsp27 in cells cultured at pH 7.3 exceed the endogenous levels in cells adapted to pH 6.7 (figure 1; also see figure 8 in ref 9). The microtubular cytoskeleton undergoes 42°C-induced rearrangement in cells cultured and heated at pH 7.3. The importance of phosphorylated Hsp27 to microtubular stability in CHO cells is unknown, and remains to be determined. As mentioned previously, phosphorylated Hsp27 plays an important role in stabilizing the microfilament system in hamster cells (28), and the actin microfilament system is stabilized against 42°C-induced rearrangements in CHO cells adapted to growth at pH 6.7 (9). Therefore, it is plausible that the protective effects of Hsp27 on microtubular stability are due to the increased, 42°C-induced levels of Hsp27 which also become phosphorylated upon heating.

Intracellular pH regulation is critical for the survival of tumor cells in acidic microenvironments of solid tumors. For cells to survive in low pH environments, they must upregulate or protect the function of proton transporters. Indeed, the intracellular pH of CHO cells cultured at pH 6.7 is maintained at similar levels as cells cultured at pH 7.3 (1,3). An intact cytoskeletal system may provide a survival advantage to tumor cells growing in an unfavorable

acidic environment by maintaining the functional integrity of proton transporters. De Ondarza and Hootman (50) have reported that an intact cytoskeleton is required for sodium-hydrogen antiporter function. Therefore, the cytoskeletal stabilization that increased Hsp27 protein levels confer to cells grown at low pH may protect the function of proton transporters during thermal stress.

Elevated levels of heat shock proteins may enable tumor cells growing under sub-optimal conditions to bypass normal cellular controls and thrive in a stressful microenvironment instead of undergoing apoptosis. As mentioned previously numerous studies from different laboratories have shown that Hsp27 (14,51-55) and Hsp70 (13,15,56-59) act as inhibitor of apoptosis proteins. Cells derived from a variety of tumors show elevated levels of one or more heat shock proteins (60-62) including Hsp27 (61-63). Therefore, heat shock proteins may protect cells from thermal stress by acting both as molecular chaperones of critical cellular structures such as the cytoskeleton and as inhibitors of apoptotic signaling.

Treatment strategies that decrease Hsp27 levels as well as other heat shock protein levels in cells overexpressing heat shock proteins could lead to an increased rate of apoptosis in tumor cells, and provide for a therapeutic gain in the clinical setting. One means of accomplishing this is to decrease the intracellular pH prior to thermal therapy by either reducing the extracellular pH (62) or inhibiting hydrogen ion transporters responsible for maintaining the intracellular pH of tumor cells in an acidic environment (64).

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Figure Legends

Figure 1. Western blot showing relative levels of hsp27 in CHO cells exposed to 37°C or 42°C.

Lanes 1 and 2, cells grown at pH 6.7 exposed to 4 nt-mismatch oligonucleotide 5'-CTCGCTGAAGATCTT-3'; Lanes 3 and 4, cells grown at pH 6.7 exposed to antisense oligonucleotide 5'-CTCGGTCATGTTCTT-3'; Lanes 5 and 6, cells grown and maintained at pH 6.7; Lanes 7 and 8, cells grown and maintained at pH 7.3. GAPDH was used as an internal control for equal loading of the lanes.

Figure 2. Demonstration of the effects of antisense oligonucleotides on the cytoskeleton of heated pH 6.7 CHO cells. Cells were heated at 42°C for 4 h or maintained at 37°C and stained for tubulin. Perinuclear collapse of the cytoskeleton is evident in the pH 7.3 grown cells and pH 6.7 grown cells treated with antisense oligonucleotides, while the cytoskeleton is resistant to collapse in cells grown and heated at 6.7. pH 7.3 grown cells (a and b); pH 6.7 grown cells (c and d); pH 6.7 grown cells treated with antisense oligonucleotides (e and f). a, c and e were maintained at 37°C while b, d and f were heated at 42°C for 4 h. Scale bar is 50µm.

Figure 3. Histograms illustrating that antisense *hsp27* oligonucleotides sensitize the microtubular CSK of pH 6.7 CHO cells to 42°C-induced reorganization. A decrease in the mean area between 37°C and 42°C samples indicates cytoskeletal collapse. (a and b) pH 7.3 grown cells; (c and d) pH 6.7 grown cells; (e and f) pH 6.7 grown cells maintained at pH 7.3 during experiment but heated at pH 6.7; (g and h) pH 6.7 grown cells treated with antisense oligonucleotides; (i and j) pH 6.7 cells treated with 4-nt mismatch oligonucleotides. a, c, e, g and i were maintained at 37°C while b, d, f, h and j

were heated at 42°C for 4 h. The mean area (Mean) \pm standard deviation (SD) and n is the number of cells sampled.

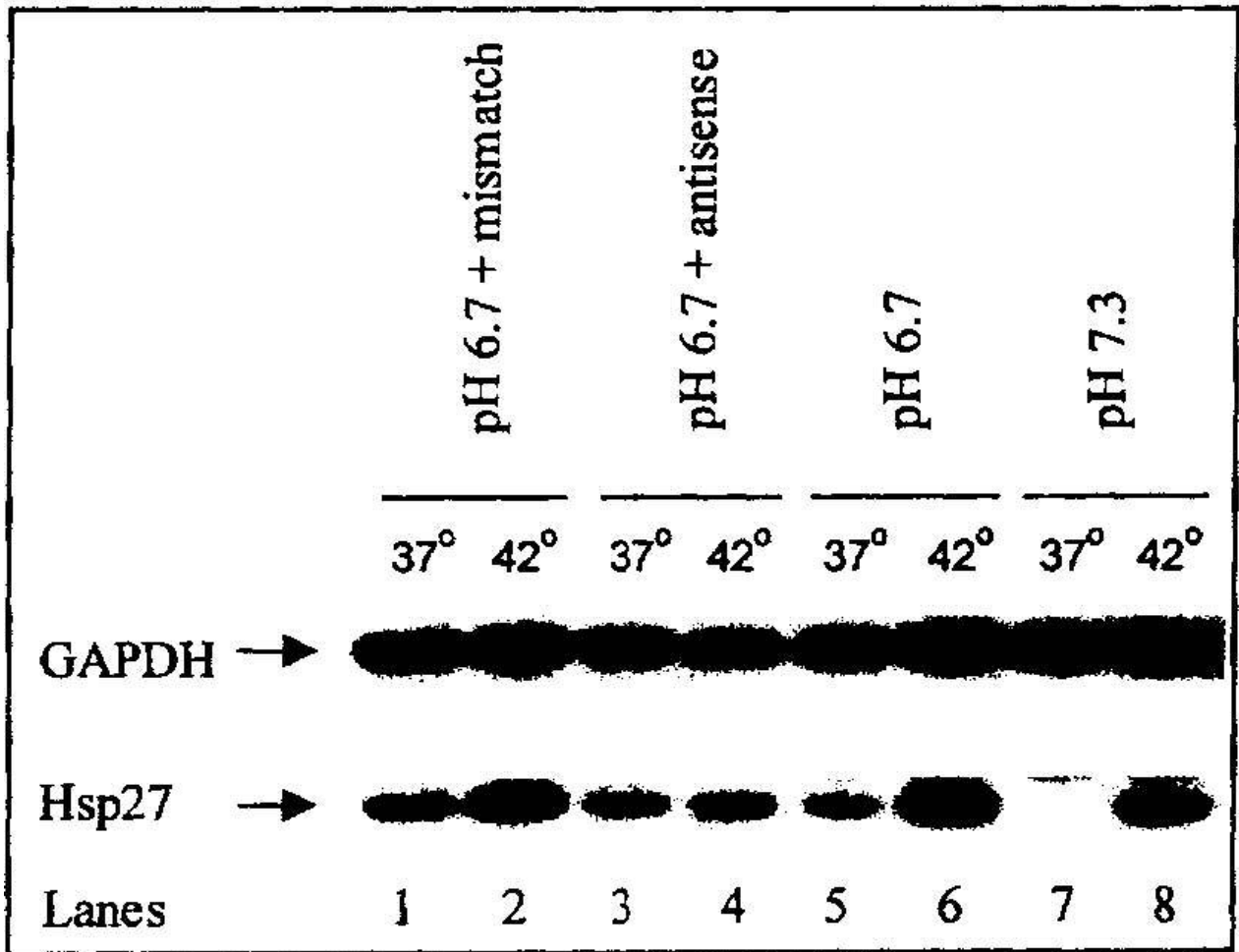


Figure 1.

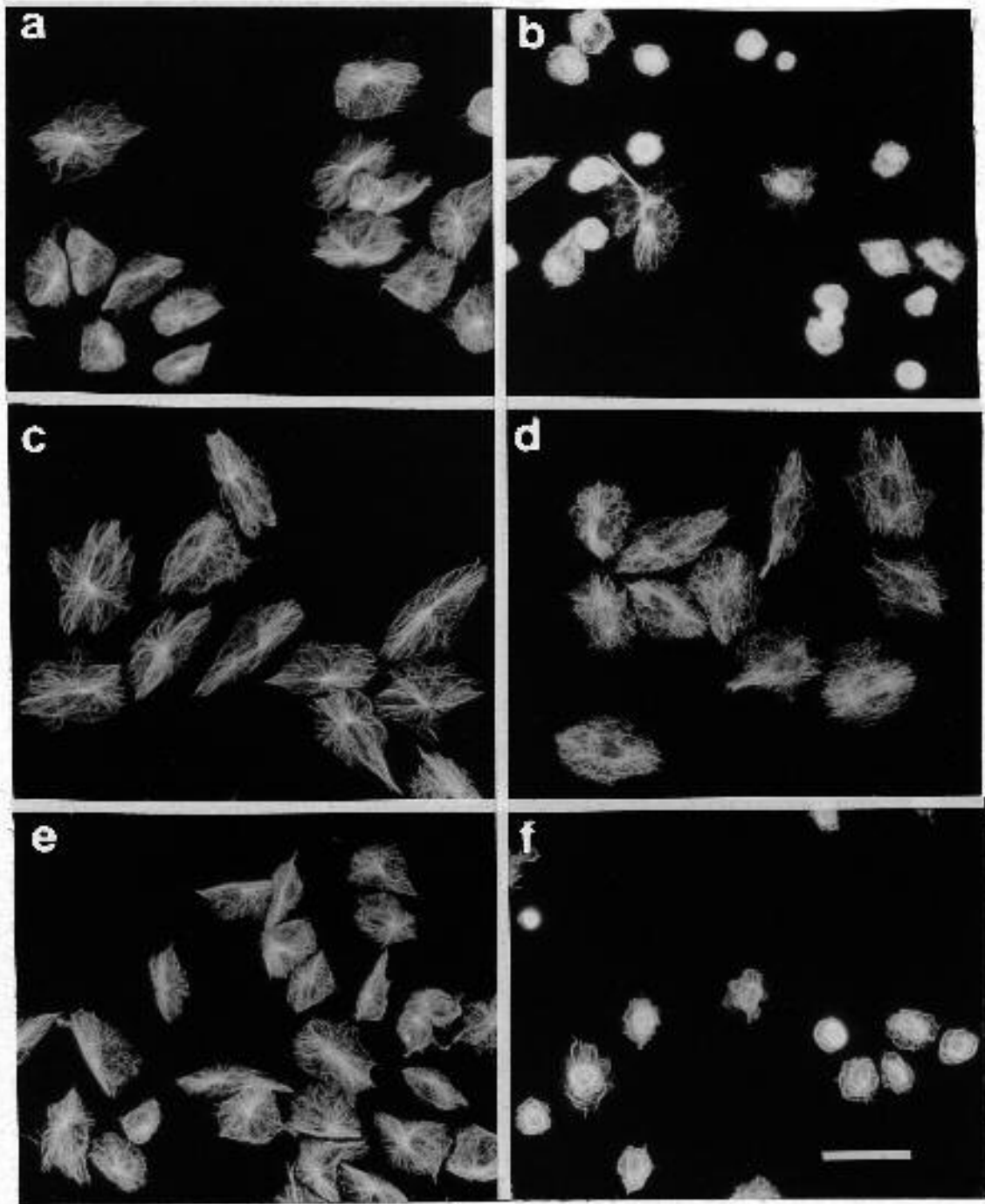


Figure 2.

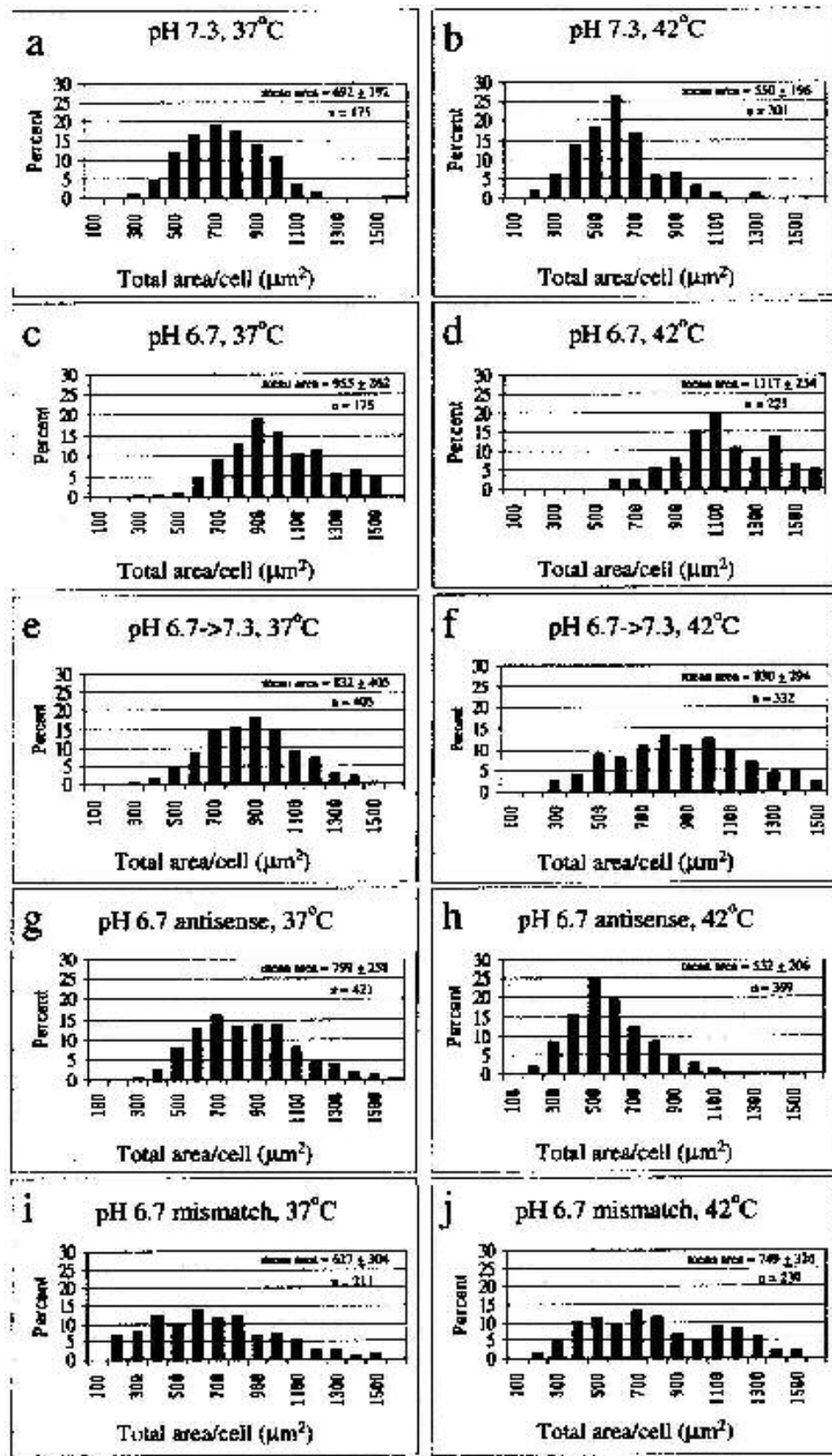


Figure 3.