Accelerated growth and senescence of arterial endothelial cells expressing the small molecular weight heat-shock protein HSP27

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ABSTRACT **Bovine arterial endothelial cells were stably transfected with the human wild-type (wt) HSP27 or a mutant gene (mu) encoding a nonphosphorylatable form of the protein. At early passage both cultural and cellular morphology were similar, although the vacuole content in wtHSP27 was much higher than muHSP27 cells. As the cultures aged, wtHSP27 cells became large, polymorphic, highly vacuolated, and reached senescence before muHSP27 transfected cultures, which remained small and polygonal with few detectable vacuoles. Vector control cells showed an intermediate phenotype. Tritiated thymidine incorporation studies were performed with multiple wtHSP27 and muHSP27 clones and the results compared with 11 vector control clones. The results showed an average increase in growth rate for the wtHSP27 cells of 3.0 ± 0.6 times. The growth rate of eight muHSP27 clones showed a slight decrease. Estradiol treatment of endothelial cells resulted in an increase in both bovine and human HSP27, with peak expression at 100 nM. Treatment of the vector-transfected cells with 100 nM estradiol resulted in a 1.44 ± 0.18 fold increase in growth rate, which was blocked by expression of muHSP27. These data demonstrate a role for HSP27 in controlling the growth rate of endothelial cells in an estrogen-responsive manner.—Piotrowicz, R. S., Weber, L. A., Hickey, E., Levin, E. G. Accelerated growth and senescence of arterial endothelial cells expressing the small molecular weight heat-shock protein HSP27.** ***FASEB J.*** **9, 1079–1084 (1995)**

Key words: wild-type HSP27 · mutant gene · estradiol treatment · Chinese hamster cells

THE HUMAN HEAT-SHOCK PROTEIN of M_r = 27 kDa (HSP27) is a ubiquitous protein with homologous analogs found in all eukaryotic cells (for a review, see ref 1). Expression and phosphorylation of HSP27 is elevated when cells are exposed to sublethal hyperthermia, although many cells constitutively express HSP27 at lower levels in the absence of stress (2). The expression of the heat-shock proteins (HSP)[2] confers thermotolerance to cells, allowing enhanced survival to subsequent challenges (3, 4).

The ability of HSP27 to act as a protective molecule during thermal stress has been demonstrated in vitro both in stable heat-resistant variants of mutagenized Chinese hamster lung cells and HSP27 transfected cells. In the former experiments, independently derived clones of the Chinese hamster cells showed constitutive overexpression of HSP27 with no change in the level of other HSPs (3). These cells showed a 5,000-fold increase in survival to a 4 h treatment of heat shock (44°C). In the latter experiments, Chinese hamster cells were transfected with the human HSP27 gene (4). The additional HSP27 provided immediate protection against heat shock and produced a permanent thermoresistance to the transfected cells.

The precise function of HSP27 in promoting thermotolerance has yet to be determined. However, during the last several years increasing evidence has supported a role for HSP27 in the regulation of actin microfilament dynamics. The first suggestion for this function was presented with the avian homologue of HSP27, which inhibited actin polymerization (5) in vitro. Transfection of Chinese hamster cells with the human HSP27 gene inhibited the disappearance of microfilaments commonly observed after heat shock. In addition, these same cells were less susceptible to actin depolymerization during acute cytochalasin D treatment and demonstrated reduced growth inhibition and death during chronic exposure to the drug (6). Overexpression of the wild-type HSP27 also increased the concentration of F-actin in the cortical filaments, generated plasma membrane ruffling, and increased pinocytotic activity. In cells containing a nonphosphorylatable mutant form of HSP27, F-actin was organized more in the stress fibers, little membrane ruffling was found, and pinocytotic activity was reduced. Therefore, HSP27 appears to play a crucial role in actin polymerization, which in turn leads to cortical microfilament assembly, membrane ruffling and pinocytosis.

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[2]Abbreviations: HSPs, heat-shock proteins; wt, wild-type; mu, mutant; DMEM, Dulbecco's modified Eagle's media; SDM, steroid-depleted media; TCA, tricholoroacetic acid; IGF, insulin-like growth factors; β-FGF, basic fibroblast growth factor; BCA, bicinchonic assay; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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To characterize the role of HSP27 in endothelial cell function, we have overexpressed both the wild-type (wt) and mutant (mu) HSP27 in bovine arterial endothelial cells (BAE) and compared growth rate with the vector-transfected control cultures. We have demonstrated that in addition to effects involving pinocytosis and F-actin organization, endothelial cell growth and morphology and senescence are also controlled in part by HSP27.

MATERIALS AND METHODS

Culture of bovine arterial endothelial cells

Low-passage bovine pulmonary arterial endothelial cells were a generous gift of W. Laug, Children's Hospital, Los Angeles, Calif. All cell culture reagents were obtained from Bio Whittaker, Inc., Walkersville, Md., except where otherwise noted. Cells were cultured under 5% CO_2 in Dulbecco's modified Eagle's media (DMEM) containing 25 mM HEPES supplemented with 10% fetal calf serum (Intergen, Purchase, N.Y.) and 1 mM each of sodium pyruvate, penicillin, streptomycin, and nonessential amino acids. Steroid-depleted media (SDM) was prepared using phenol red-free DMEM (Sigma Chemical Co., St. Louis, Mo.) and charcoal-filtered fetal calf sera (7). Cell numbers of cultures being passaged were obtained using an automated counter (Coulter Electronics, Miami, Fla.). Cells were plated at a density of $0.7–2 \times 10^4$ cells/cm^2 and passaged when confluent (approximately 1×10^5/cm^2).

Plasmids

pHS2711 is a genomic clone of human HSP27, and p157882 contains a human HSP27 gene in which the codons for Ser^{15,78,82} were converted to glycine (nonphosphorylatable). Plasmid pBS was used in the vector controls. Plasmid CDM8^{neo} contains the neomycin (Geneticin) resistance gene and was a gift from Dr. Jerry Ware, The Scripps Research Institute, La Jolla, Calif.

Transfection, selection, and cloning

Passage 4-6 BAE cells were plated at a density of $0.7–1.1 \times 10^4$/cm^2 in 9 cm^2 culture wells, cultured for 12–16 h, and then transfected using Lipofectamine reagent (Gibco-BRL, Gaithersburg, Md.). Cells were transfected with 0.2 μg of pCDM8^{neo} and 2 μg of either pHS2711, p157882, or pBS in 200 μl serum free media containing 160 μg/ml Lipofectamine for 6 h at 37°C; the cells were cultured in complete media for 72 h, duplicate wells were harvested for immunofluorescence and Western blotting to determine relative transfection efficiencies. Remaining wells were then cultured in selection media (complete media containing 700 μg/ml Geneticin; Gibco-BRL) and stably transfected cultures were established. Cells were cloned in 0.2 cm^2 wells by limiting dilution in cloning media (selection media of which 25% had been conditioned by growing BAE cultures). Cloning wells containing a single cluster were passaged 2–3 wk after plating and human HSP27 expression was subsequently monitored via immunoblotting.

Determination of F-actin ratios

Relative F-actin content was measured by fluorescein-phalloidin staining of the wt, muHSP27, and vector control cultures followed by extraction and spectrofluorometric analysis. Cells were fixed with 3.7% paraformaldehyde for 10 min at room temperature, washed with PBS, and permeabilized with 0.5% Triton X-100/PBS for 10 min. Fluorescein-phalloidin (4 units/ml in PBS; 0.5 ml/well; Molecular Probes, Eugene, Oreg.) was added and incubated at room temperature in the dark for 1 h. Each well was washed three times with PBS and the fluorescein-phalloidin was extracted with 100% methanol for 1 h. The extracts were analyzed spectrofluorometrically with excitation and emission wavelengths of 450 nm and 545 nm, respectively. Results are presented as a ratio of wt or muHSP27 cell to vector control.

SDS-PAGE, immunoblot analysis, and isoelectric focusing

Transfected cells were grown in 9 cm^2 wells to confluence, rinsed with DPBS, and then lysed in 200 μl SDS-PAGE sample buffer (2% SDS, 50 mM Tris, pH 6.8, 10% glycerol, and 0.1% bromophenol blue). Total protein content of the lysates was determined using the bicinchonic assay (BCA, Peirce Chemical Co., Rockford, Il.). Twenty micrograms of each lysate was then reduced with 5% 2-mercaptoethanol, boiled for 3 min, and subjected to SDS-PAGE in 12% (w/v) acrylamide gels according to the procedure of Laemmli (8). One microgram of recombinant human HSP27 (StressGen, Victoria, B.C., Canada) was included in each gel.

For immunoblot analysis, the proteins were transferred to 0.2 μm nitrocellulose membranes as described (9); the membranes were blocked for 1 h at room temperature in 3% nonfat milk and then incubated with 2 mg/ml of G3.1 monoclonal anti-human HSP27 or rabbit anti-murine HSP27 (StressGen) in 50 mM Tris, pH 7.4, 145 mM NaCl, 0.05% Tween 20, 1 mg/ml BSA, and 0.1% sodium azide (TTBS-BSA) overnight. Bound primary antibody was detected using goat anti-mouse or rabbit IgG conjugated to biotin, horseradish peroxidase-conjugated streptavidin (Zymed Lab., San Francisco, Calif.), and the ECL chemiluminescence reagent (Amersham, Arlington Heights, Il.). For isoelectric focusing the cell extracts were diluted 1:3 in lysis buffer containing 8 M urea, 8% NP-40, 5% b-mercaptoethanol, and 4% ampholines pH 5-8 (Pharmacia, Biotech, Piscataway, N.J.). As described previously (10), the samples were focused in 3% acrylamide gels containing 8% NP-40, 8 M urea, and 4% ampholines and then transferred to a 12% SDS-PAGE acrylamide gel for second-dimension separation.

Labeling with $^{32}P_i$

Cells were cultured in complete media prepared with phosphate-free DMEM (Sigma) containing 100 μCi/ml of ^{32}P-labeled orthophosphate (Dupont/NEN, Richfield, Il; 900 Ci/mmol) for 45 min at 37°C. At this point, 100 nM phorbol, 12-myristate, 13-acetate (Sigma) or vehicle was added directly to the cultures and incubated for an additional 10 min. The cells were then rinsed three times with DPBS and prepared for SDS-PAGE. Upon completion of electrophoresis, the gels were stained with 0.125% Coommassie brilliant blue R-250 (BioRad, Richmond, Calif.) in 50% methanol, 10% acetic acid for 10 min, destained, and subjected to autoradiography at -80°C using Kodak XAR X-ray film (Rochester, N.Y.).

[^{3}H]Thymidine Incorporation and cell counting

The growth of subconfluent cultures of transfected cells was followed by measuring [^{3}H]methylthymidine incorporation. Cells were plated in 9 cm^2 wells at 8000/cm^2 and cultured for 2 to 3 days, with fresh media added daily. One μCi/ml of [^{3}H]methylthymidine (Amersham, Inc.) in fresh media was added to the culture wells. After 2 h the media were removed and the cells were rinsed three times with DPBS. Well contents were precipitated with 10% trichloroacetic acid (TCA) for 5 min. Unincorporated [^{3}H]methylthymidine was removed with one wash of 10% TCA and one wash with deionized water. The contents of each well were then solubilized in 0.5 ml of 0.5 N NaOH for 10 min. Fifty microliters of each sample was neutralized with 0.5 N HCl and assayed for total protein concentration using the BCA assay. Radioactivity on the remaining portion of the samples was measured and the amount of incorporated [^{3}H]methylthymidine was normalized for cellular protein (cpm/mg). Final values ranged from 90 to 227 cpm/μg protein and 274 to 817 cpm/μg protein in extracts from vector containing cells and wtHSP27 expressing cells, respectively. Total number of counts detected in the various samples was between 19,800 to 78,000 cpm.

RESULTS

Transfection of bovine endothelial cells with human HSP27

Bovine pulmonary endothelial cells were transfected with either the wt or muHSP27 genomic clones and the expression of the protein evaluated by immunoblot analysis. Two differences between the bovine and human HSPs become evident in these results (**Fig. 1**): *1)* the bovine protein is smaller than the human and has a molecular weight (25,000 kDa) identical to that of the rodent HSP25 (11)), and *2)* the bovine HSP exhibits a greater avidity for anti-sera raised to murine HSP25 than the human HSP but a weaker activity for the monoclonal antibody raised to human HSP27. The latter observations suggest that the bovine HSP25 shares a greater number of antigenic determinants with murine HSP25 and is therefore more homologous with the murine protein. Represented in Fig. 1 are examples of the multiple clones isolated from the wt and muHSP27 and the vector-transfected cultures showing the different expression levels of the human protein and the stable level of endogenous HSP in all of the transfected and nontransfected cells.

Figure 1. Bovine endothelial cells transfected with human HSP27. Duplicate immunoblots of extracts from cells transfected with: (lanes) 1, nothing (native); 2, pBS vector only; 3 and 4, wtHSP27 gene; 5 and 6, muHSP27 gene. Lanes 2–6 are independently isolated clones. *A)* Immunostained with mouse anti-human HSP27. *B)* Immunostained with rabbit anti-rodent HSP27.

Comparison of the phenotypic characteristics of wt, muHSP27, and vector-transfected cultures demonstrates dramatic differences emerging during long-term culture with respect to cell morphology and vacuole content. The three cultures shown in **Fig. 2** (transfected at passage 8) were treated identically after clonal isolation; all were grown to confluence after each passage and then replated in parallel at the same cell density (Fig. 2). At passage 12 the cultures had a similar morphology with compact, polygonally shaped cells forming a cobblestone monolayer (panels *A, B, C*). At later passage (passage 16), however, the wtHSP27 expressing cells became enlarged, polymorphic, and contain large vacuoles (Fig. 2*E*) whereas the muHSP27 cells retained the same morphology as the younger cultures (panel *F* vs. panel *C*). The vector control shows an intermediate morphology with a number of larger cells now present and both large and smaller cells containing vacuoles (Fig. 2*D*). The results were consistent among clones and antibiotic selected, mixed populations of transfected cells. As the cultures were passaged further, it became apparent that the enlarged vacuole-laden phenotype of the wtHSP27 portended the onset of senescence. Cultures containing the wtHSP27 became senescent and were no longer capable of surviving continued subculture well before the muHSP27 cells. The wt clone represented in Fig. 2 became senescent at passage 18 whereas the muHSP27 clone has survived past passage 25. Thus, expression of the wt HSP27 leads to a pronounced acceleration of the senescent phenotype whereas muHSP27 expression maintains the characteristic vascular endothelial cell phenotype. The effect of wt and muHSP27 on actin filament organization described previously was also observed in these endothelial cells.

Bovine endothelial cells expressing human HSP27 exhibit faster growth

During the cloning of the wt and muHSP27 transfectants, it became apparent that the wtHSP27 cells grew at a faster rate than the mutant or the vector control. To establish whether this perceived difference had a basis in fact, nine clones of wtHSP27 expressing cells and 11 vector controls were repeatedly subcultured and growth rate was determined by [^{3}H]thymidine incorporation rates. For each clone, the amount of radiolabel incorporated (normalized for protein content) was divided by the mean value obtained from the 11 vector control clones. The average growth rate for each clone (5 data points/clone over a 1 month period; **Fig. 3**) ranged from 2.3 to 3.7 times the mean growth rate of the vector controls, with an average increase of 3.0 $\pm$ 0.6 for all nine clones. Single data point experiments with other panels of clones (18 additional clones) developed from different transfections generated similar results (2.3 $\pm$ 0.9; range = 1.3 to 4 times). Clones expressing the muHSP27 showed no significant difference in the growth rate com-

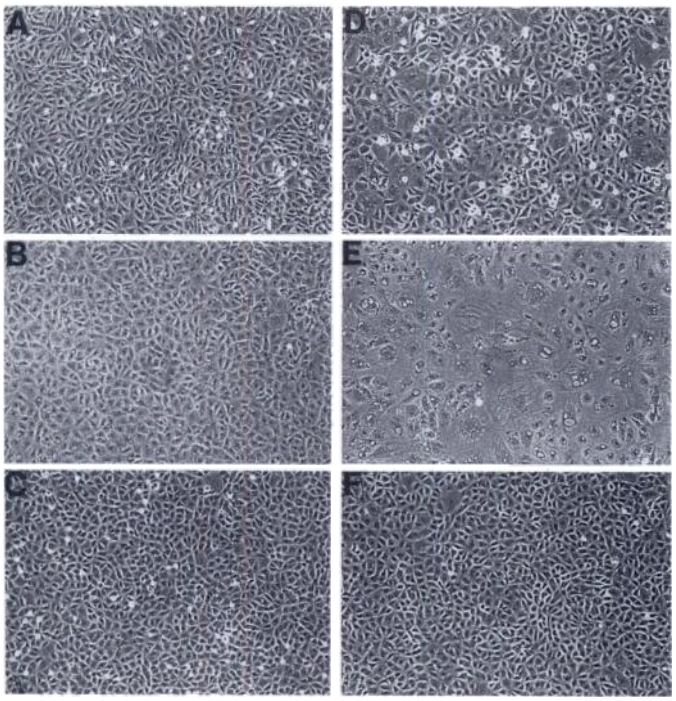

Figure 2. Cultural morphology of vector, wtHSP27, and muHSP27 transfected endothelial cells. Cells were transfected at passage 6 and treated identically at each passage. *A, B, C)* Passage 12; passage 16 *D, E, F);* vector control *A, C);* wtHSP27 *B, D);* muHSP27 *C, F).* Magnification ×100.

pared to the vector controls. The relative rate of growth was 1.1 ± 0.3 (n = 16; eight different clones) even though the muHSP27 clones used had similar levels of human HSP27 to that on the wtHSP27 expressing clones.

Expression of transfected human HSP27 and cell growth is responsive to estradiol

Treatment of estrogen-dependent tumor cells with estradiol results in a concomitant increase in HSP27 expression and tumor cell growth (12, 13). To determine whether the genomic clones transfected into the endothelial cells would respond to β-estradiol and whether increased expression would affect growth rate, the cells were cultured in SDM prepared with phenol red free DMEM and supplemented with charcoal-stripped fetal calf sera. Analysis of HSP27 antigen levels in cultures treated with increasing concentrations of β-estradiol showed a dose-dependent increase in HSP27 levels with the maximum response at 100 nM (**Fig. 4**). This response to the different concentrations of estradiol was the same with wtHSP27, muHSP27, and the native protein (wtHSP27 is shown in Fig. 4; both wt and muHSP27 gave identical results). Treatment with 1 μM estrone, which exhibits a much lower affinity for the estrogen receptor, had no effect on HSP27 levels (data not shown).

To determine whether the growth rate of endothelial cells not expressing human HSP27 is modified during stimulation of the endogenous protein alone, the effect of estradiol treatment on growth rate of cells transfected with the vector only was examined. Cultures were incubated in SDM for 72 h in the presence of 100 nM estradiol and the growth rate was determined. The growth rate in SDM in the absence of estradiol was similar to that in complete medium (ratio complete medium:SDM = 0.91 ± 0.1; n = 9). In the presence of the steroid, however, growth rate increased by 1.44 ± 0.18 times (n = 3). This enhancement of growth was observed regardless of the basal rate (minus estradiol) of the individual clones. In contrast, when the experiments were repeated with cells expressing the muHSP27, no increase in growth was observed (0.90 ± 0.11; n = 3).

DISCUSSION

Overexpression of the small molecular weight heat-shock protein HSP27 in fibroblasts stabilizes microfilaments during heat stress and cytochalasin B treatment, promotes increased pinocytotic activity, reorganization of actin filaments into the dense peripheral bands, and membrane ruffling (14) whereas the nonphosphorylatable mutant HSP27 has a negative effect on these same processes. From these data and previous studies that showed possible HSP27 control of actin polymerization it has been suggested that this protein is one of the components controlling actin microfilament dynamics. We have now demonstrated that overexpression of HSP27 in bovine pulmonary endothelial cells stimulates the growth rate of these cells and accelerates the rate at which the cultures reach senescence.

There have been several reports in which increased expression of HSP27 has been associated with rapid or persistent cell growth, e.g., during fetal development and tumor growth, although no direct link between the two has been established (1). The data presented here relate the enhanced expression of wtHSP27 in aortic endothelial cells

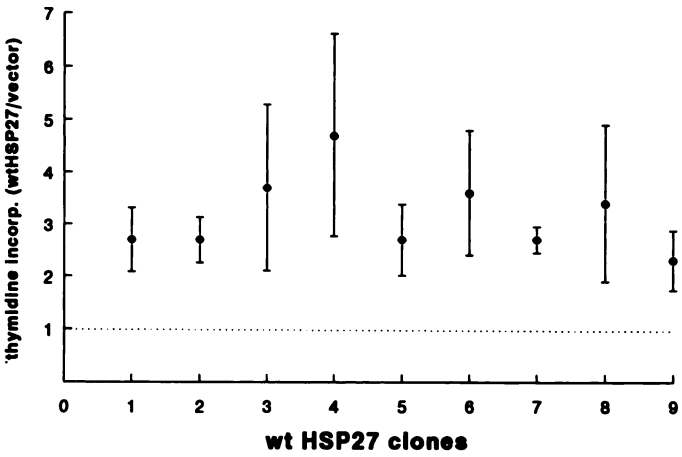

Figure 3. Thymidine incorporation studies: wt HSP27 clones vs. vector controls. Nine wtHSP27 clones and 11 vector controls were repeatedly passaged for 1 month (five times) and thymidine incorporation studies were performed during the growth phase of each passage. The result for each wtHSP27 clone represents the average of the five experiments and is presented as the ratio of label incorporated into each clone (cpm/μg protein) divided by the mean value obtained from the panel of 11 vector transfected clones.

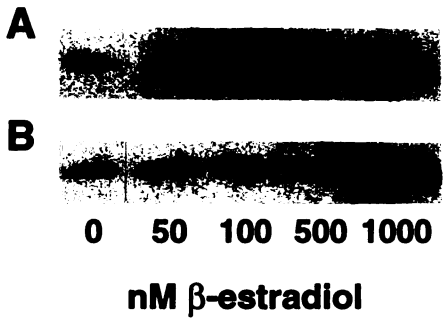

Figure 4. Induction of native and transfected HSP27 by estradiol treatment. Vector control or human wtHSP27 and muHSP27 expressing cells were treated with increasing concentrations of β-estradiol for 24 h and the levels of HSP27 were determined by immunoblot analysis. *A*) Extracts from wtHSP27 containing cells probed with anti-human HSP27; *B*) extracts from vector control cells probed with anti-rodent antibodies.

with an average two- to threefold increase in growth rate. This accelerated growth is not a function of specific cloned cultures, which do tend to display heterogeneous behavior, but is observed in all of the clones tested albeit to different degrees. Even the cells affected the least displayed a 1.5-fold increase over the vector controls. Expression of the mutant to levels equal to that of the wtHSP27 did not increase the growth rate, indicating that the effect is specific for fully functional HSP27 and that phosphorylation is required. However, muHSP27 failed to reduce the growth rate compared to the family of vector control clones; an average of only 10% change in growth rate was not statistically significant. In fibroblasts, muHSP27 has been reported to act as a dominant negative mutant of HSP27 function. The difference between the two observations could be due to the ratio of muHSP27 to the endogenous protein, which may be too small in these clones to show a prominent dominant negative affect. The failure of the muHSP27 to inhibit growth may also be a function of a more fundamental problem of cell survival, as suggested by the limited number of muHSP27 clones that were generated during clonal isolation. From the five transfections performed, between 100 and 200 wtHSP27 expressing clones and vector controls were isolated, but only 12 mutant containing clones. The difficulty in generating muHSP27 was a result of low cell survival during the cloning process. Even though transfection efficiency was the same in all three cases (5–10%), few of the muHSP27-containing cells surviving antibiotic selection were able to form colonies. Therefore, the overexpression of muHSP27 appears to have a negative effect on the ability of endothelial cells to recover from and survive the stress of antibiotic selection. Whatever the cause, the important result in these studies is that without the appropriate phosphorylation sites HSP27 does not play a role in promoting growth.

The increase in growth rate in response to estradiol and its abrogation by muHSP27 indicate that the growth-related effect we observed with wtHSP27 could represent an endogenous pathway that is controlled by environmental mediators through HSP27 expression (12, 15). As has been reported earlier, HSP27 expression is responsive to estradiol in tumor cells; in fact, this protein was first identified as an estrogen receptor-related protein in mammary carcinoma cells and an estrogen-responsive element has been identified in its 5′ promoter region (16). The stimulation of mRNA and HSP27 antigen levels of the endogenous HSP27 and the transfected genomic clones of human HSP27 in endothelial cells therefore is not unexpected.

The mitogenic effect of β-estradiol on BAE cells may be similar to that of estrogen-responsive tumor cell lines (MCF-7 cells) where β-estradiol also induces HSP27 expression and exerts a mitogenic effect (12). The proliferative effect of β-estradiol on these cells is attributed to several phenomena, the relative contributions of which are of some debate. First, β-estradiol has a sensitization effect; i.e., it makes the cells more responsive to the proliferative effect of insulin-like growth factors (IGF), basic fibroblast growth factor (β-FGF), transforming growth factor α (TGFα) and EGF (17). For example, cells exposed to β-estradiol exhibit a greater propensity to duplicate in response to a minimal β-FGF stimulus (18). Second, the proliferative effect of β-estradiol may be due to the induction of autocrine growth factors (7). Increased expression of HSP27 by estradiol may represent one of the pathways by which the cells became more responsive to growth factors, working in concert to facilitate conversion of the extracellular signal to the intracellular changes necessary for cell replication. The accelerated growth that has been associated with expression of HSP27 may be relevant to angiogenesis in environments where stimulatory factors like estrogen are a key regulatory molecule; i.e., the vascularization of tumors of estrogen-targeted tissues (19), the complex angiogenic process of uterine artery growth during the proliferative phase of the menstrual cycle (20), and neovascularization during embryonic development (2).

The mechanism of the accelerated growth may be related to actin filament dynamics, which is undoubtedly an important factor in cell division and growth control. Nontransformed mammalian cell shape is tightly coupled to DNA synthesis, with flat cells incorporating thymidine at a greater rate than more spherical cells (21, 22). Cell shape is determined by multiple factors, one of the most important being actin microfilament organization, a process that is likely to involve HSP27 expression and/or phosphorylation. Another possibility for HSP27 control of cell growth is the role actin plays in cytokinesis. Cleavage in animal cells involves constriction of the contractile ring or arc, both of which are composed of actin filaments, and it has been proposed that actin-modulating proteins are involved in the process of contractile ring organization and disintegration.

The promotion of cell senescence by wtHSP27 is a dramatic and probably extreme example of the effect elevated HSP27 can have on endothelial cell integrity. The cause (or causes) of the accelerated senescence or the maintenance of cell viability may be multifactorial and result from the other effects of HSP27. For example, elevated pinocytotic

activity over prolonged periods of time may overwhelm the ability of the cells to process, degrade, and/or exocytose media components, whereas the reduction in vesicle formation in muHSP27 cells may slow that process. Alternatively, the accelerated growth rate and the increased number of cell divisions (in contrast to the passage number) may generate an older population of cells more quickly in the wtHSP27-containing cells. In either case, it is apparent that elevated levels of HSP27 or its dominant negative mutant have profound effects on endothelial cell behavior that can be traced to the proteins proposed interaction with the cytoskeleton. FJ

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Received for publication May 5, 1995.

Received for publication May 12, 1995.