

The Small Heat Shock Protein hsp27 Is Correlated with Growth and Drug Resistance in Human Breast Cancer Cell Lines¹

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Abstract

An emerging body of evidence suggests that the heat shock proteins (hsp) may be involved in drug resistance. When hsp are induced by elevated temperatures, resistance to doxorubicin (Dox), but not to other commonly used chemotherapeutic agents, is induced in breast cancer cells. To evaluate the role of hsp27 in this phenomenon, we have transfected MDA-MB-231 breast cancer cells, which normally express low levels of hsp27, with a full-length hsp27 construct. These hsp27-overexpressing cells now display a 3-fold elevated resistance to Dox. Anchorage-dependent proliferation and anchorage-independent growth were also increased 2-4-fold in these transfectants. We have also derived a MCF-7 breast cancer cell line with amplified endogenous hsp27 which is highly resistant to Dox. When these cells are transfected with an antisense hsp27 construct, they are rendered sensitive to Dox (3-fold) with anchorage-dependent as well as anchorage-independent growth, similarly decreased. These results suggest that hsp27 specifically confers Dox resistance in human breast cancer cells and, furthermore, that hsp27 may be involved in the regulation of cell growth.

Introduction

The heat shock proteins are thought to protect cells from a variety of external toxic stimuli. It is known that expression of one of these proteins, hsp27,⁴ is low in normal human breast tissue and that it is variably expressed in benign breast lesions. In contrast, hsp27 is often found at high levels in human breast tumor cells (1), and, although data are conflicting, levels of expression may have prognostic importance in breast cancer (1, 2). Recent data also strongly suggest that hsp27, along with another protein, hsp70, may contribute to cellular resistance to cytotoxic drugs, particularly Dox (3). In this study, breast cancer cell lines were exposed to heat shock treatment followed by Dox exposure with the resulting elevated hsp27 and hsp70 levels associated with Dox resistance. Sensitivity to other drugs such as cholchicine and methotrexate was not changed by heat treatment in these cells. Since the chemotherapeutic treatment of breast cancer is often hindered by both intrinsic drug resistance and the development of acquired drug resistance during treatment (4), and since Dox is the most active single agent currently used in the treatment of breast cancer, the finding of specific Dox resistance in breast cancer cells subsequent to heat shock treatment suggests that hsp expression in general, and perhaps hsp27 expression specifically, could be very important clinically. We have derived an in vitro model to test whether

the small heat shock protein hsp27 is *directly* involved in the phenomenon of heat-induced Dox resistance. hsp27 expression vectors were introduced into both the estrogen receptor-negative cell line MDA-MB-231 which expresses low endogenous levels of hsp27 and into the estrogen receptor-positive MCF-7/MG cell line which expresses high levels of hsp27 due to gene amplification.⁵ We transfected these cells with a full-length human hsp27 cDNA in either the sense (MDA-MB-231) or the antisense (MCF-7/MG) orientation and asked whether these cells were now more resistant or sensitive, respectively, to Dox treatment. Because hsp may also regulate cell proliferation (5, 6) and since cell cycle transit may influence sensitivity to cytotoxic agents, we also investigated the effects of hsp expression on anchorage-dependent and -independent cell growth.

Materials and Methods

Cell Culture and Maintenance. The breast cancer cell line MDA-MB-231 was obtained from the American Type Culture Collection (Rockville, MD). MCF-7/MG cells were originally obtained from Dr. Herbert Soule (Michigan Cancer Foundation) and have been maintained in our laboratory for the past 10 years. These cell lines were maintained in Eagle's medium supplemented with 10% fetal bovine serum, 6 ng/ml insulin, and 25 µg/ml gentamicin sulfate (Schering Corporation, Kenilworth, NJ) and were cultured in T-75 flasks as continuous monolayer cell lines. Cells were periodically tested for Mycoplasma contamination (Bionique Laboratories, Saranac Lake, NY).

Preparation of hsp27 Expression Vector and Transfection Analysis. A full-length human hsp27 cDNA was prepared by PCR amplification using a sense primer (5'-GCGAATTCCTGAGCAGACGTCCAGAGCA-3') and an antisense primer (5'-CGGAATTCGCATCCGGGCTAAGGCTTT-3') corresponding to nucleotides -32 to -13 and 617 to 635, respectively, with *EcoRI* sites plus 2 additional nucleotides added to the 5' end of each primer (7). PCR reactions were carried out as described (8), and the fragments were cloned into the *EcoRI* site of pGEM7zf+ (Promega, Madison, WI). The *EcoRI* fragment was amplified and then subcloned into the *EcoRI* site of the mammalian expression vector pMTP-5H (9) which is under the control of a cadmium-inducible metallothionein promoter. hsp27, either in the sense or the antisense orientation relative to the metallothionein promoter, was then cotransfected with the G418-selectable expression vector pSVneo into MDA-MB-231 or MCF-7/MG cells, respectively, at a molar ratio of 25:1. Control transfections were also done with the pMTP-5H vector alone plus pSVneo in all experiments. The Lipofectin transfection method was followed according to the manufacturer's recommended procedure (GIBCO BRL, Grand Island, NY). After 16 h in serum-free Opti-Mem medium (GIBCO BRL) the cells were washed, and 1 day later they were split into four 15-cm dishes in Dulbecco's modified Eagle's medium (10% fetal bovine serum) containing 600 µg/ml (MDA-MB-231) or 800 µg/ml (MCF-7/MG) of G418. The medium was changed every 3-4 days and colonies were picked approximately 21 days later.

RNA Analysis. To analyze for changes in hsp27 mRNA levels in transfected cells, total cellular RNA was extracted on an Applied Biosystems Model 340A nucleic acid extractor following the manufacturer's recommendations (Applied Biosystems Inc., Foster City, CA). A 350-base pair hsp27 fragment was amplified by PCR using primer the sense hsp27 and another antisense primer (5'-GGCGAAGTGGTTGACATCCA-3') corresponding to nucleotides 295 to 315 (7) and then subcloned into the *EcoRI/Kpn* site of pGEM7zf+. An RNase protection assay probe was then synthesized from the 350-base pair

Received 6/7/93; accepted 8/18/93.

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¹ This work was supported by NIH Grant CA11378, a Cancer Center support grant from the National Cancer Institute CA 54174, and NIH Grant CA58183-01.

² S. O. is a recipient of a postdoctoral fellowship from Deutscher Akademischer Austauschdienst and from the Susan G. Komen Foundation.

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⁴ The abbreviations used are: hsp, heat shock protein; Dox, doxorubicin; IC₅₀, drug concentration(s) inhibiting colony formation by 50%; Tf., transfectants; cDNA, complementary DNA; PCR, polymerase chain reaction; Cd, cadmium chloride; ANOVA, analysis of variance; CHO, Chinese hamster ovary; mdr, multidrug resistance.

⁵ S. Fuqua, unpublished results.

hsp27 fragment with a Ribprobe kit (Promega, Madison, WI) using 50 μ Ci [32 P]CTP. As previously described (3), the 36B4 control probe (digested with *Rsa*I) was used to demonstrate equivalent RNA loading. RNase protection assays were performed with 30 μ g of total RNA from the transfectants (3) and the gels were exposed to X-OMAT X-ray film (Kodak, Rochester, NY) overnight, and quantitated by densitometry on a DU-7 Spectrophotometer (Beckman, Fullerton, CA), using the area of the 36B4 band for normalization of the hsp27 signals.

Protein Analysis. Cells were mechanically harvested to prevent endogenous induction of hsp and washed in phosphate-buffered saline, and a 5% sodium dodecyl sulfate solubilized cellular extract was prepared as previously described (10). After protein determination by the bicinchoninic acid method (11) (Pierce, Rockford, IL), equal amounts of protein were resolved according to the Laemmli technique (12) on a 12.5% polyacrylamide gel. After electrophoresis, proteins were transferred from the gel to nitrocellulose membranes and subjected to immunodetection using an hsp27-specific antibody developed in our laboratory (13), and 125 I-labeled sheep anti-mouse IgG (100,000 cpm/ml; Amersham, Arlington Heights, IL). The Western blots were then exposed for 1–3 days to X-ray film at -70°C using intensifying screens.

Growth Analyses. To analyze anchorage-dependent growth, cells were first plated at day 0 at a density of $1.5 \times 10^4/\text{cm}^2$ in 12-well plates (Falcon,

Lincoln Park, NY). In specific experiments, after 24 h, Cd was added to a final concentration of 1 μM for 6 h, the Cd was then removed, and the medium was changed every other day. After harvesting on day 2, 3, and 6, the cells were counted and the results represented as the mean values of 8 independent determinations of the cell number for each time point in 2 separate experiments. Clonogenic assays were also performed to assess anchorage-independent growth (3). Cells were first grown in T-25 flasks and were used when approximately 70% confluent. Untreated or 1 μM Cd-treated cells were pelleted, washed in phosphate-buffered saline, resuspended, and plated as described previously (3). For drug resistance experiments, cells transfected with vector alone or with hsp27 cDNAs were exposed before plating to varying concentrations (0.01–1.0 $\mu\text{g}/\text{ml}$) of doxorubicin hydrochloride (Hoffmann-LaRoche Inc., Nutley, NJ) at 37°C for 1 h; all experiments were done in duplicate.

Statistical Methods. Anchorage-independent growth of the hsp27 transfectants and their associated controls were compared using two sample *t* tests. Anchorage-dependent growth was observed at several time points and the growth of control and hsp27 transfectants was compared using two-way ANOVA with one factor for the time effect and one factor for the effect of hsp27. For each cell line, the effect of hsp27 on Dox resistance was evaluated by comparing log transformed IC_{50} for transfectants and their associated con-

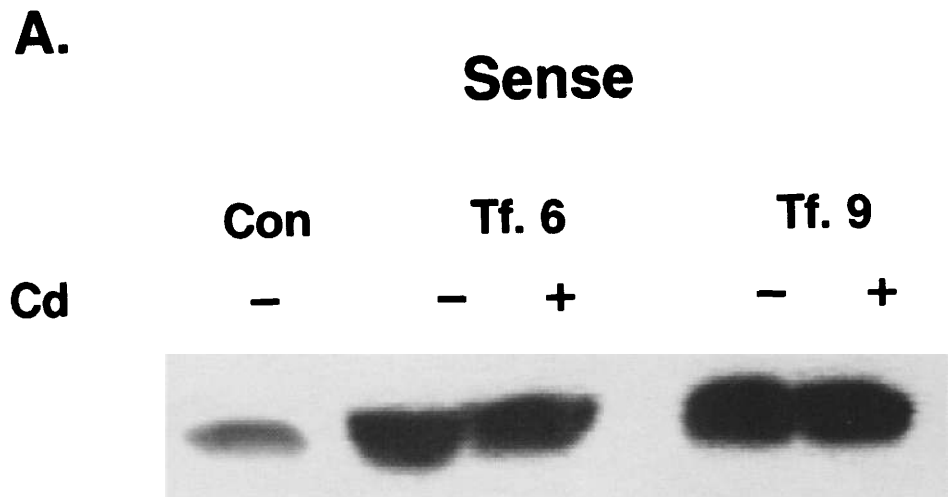
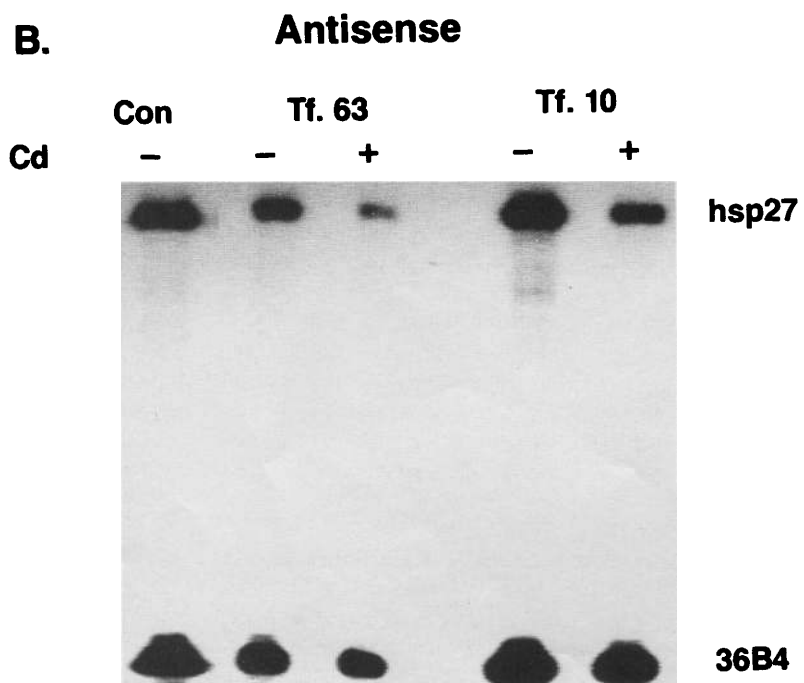


Fig. 1. *A*, overexpression of hsp27 in MDA-MB-231 cells as determined by Western blot analysis. The results of two independent clones (Tf.6 and Tf.9) are shown. *B*, reduction of endogenous hsp27 mRNA in MCF-7/MG cells as determined by RNase protection assay. The positions of hsp27 and 36B4 are indicated. The results of two independent clones (Tf.63 and Tf.10) are shown. Cd was added as indicated.



trols in a repeated measures ANOVA in which clone was included as a blocking factor and there were 2–4 growth experiments/clone. IC_{50} were obtained from smoothed growth inhibition curves for each experiment. Fold changes in resistance to Dox and 95% confidence intervals were estimated by back-transforming means and 95% confidence intervals of differences in log-transformed IC_{50} . The resulting estimates are consistent with the ANOVA used to test significance and are more stable and conservative than simple averages of ratios of IC_{50} . All analyses were performed using SAS version 6 (SAS Institute, Inc., Cary, NC)

Results

Characterization of Transfected Clones. We used the pMTP-5H expression vector to drive hsp27 transcription because we had previously determined that in both of the parent cell lines (MDA-MB-231 and MCF-7/MG), neither endogenous hsp27 mRNA nor protein levels were influenced by Cd at the concentrations used for induction of hsp27 with this vector system (data not shown).

MDA-MB-231, which expresses a low endogenous level of hsp27, was cotransfected with plasmids expressing hsp27 in the sense orientation and G418 resistance. Out of 11 independent clones tested, 9 G418-resistant clones carried the hsp27 insert as determined by PCR amplification (results not shown). These nine PCR-positive clones were then tested for hsp27 mRNA expression by RNase protection assay (results not shown); the two highest hsp27-expressing, stably transfected cell lines Tf 6 and Tf.9 were then subjected to Western blot analysis with an hsp27-specific antibody (Fig. 1A) to determine hsp27 steady-state levels in these two transfectants. Tf.6 and Tf.9 demonstrated a 6–8-fold overexpression, respectively, of hsp27 as determined by densitometric scanning of the Western blot. Additionally, both of these clones exhibited constitutive overexpression of the introduced sense hsp27 construct, since Cd did not further increase hsp27 levels in these cells (Fig. 1A, +/- Cd). Therefore all further experiments with these two clones were conducted in the absence of Cd.

In contrast to the transfection of hsp27 in the sense orientation, the transfection efficiency of antisense hsp27 into MCF-7/MG cells was much lower. Out of 65 clones tested by DNA PCR for introduction of hsp27, only 8 were positive for hsp27 vector insertion. Fig. 1B demonstrates the down-regulation of endogenous hsp27 mRNA levels in two independent antisense hsp27 clones. We utilized RNase protection assays to determine whether endogenous levels of hsp27, normally high in MCF-7/MG cells, were decreased upon induced antisense hsp27 expression (+ Cd) in these two clones. In contrast to that seen with the sense hsp27 transfectants, antisense hsp27 remained inducible by Cd in the MCF-7/MG transfectants (2 are shown in Fig. 1B, Tf.63 and Tf.10). Tf. clone 63 exhibited the greatest level of inhibition of endogenous hsp27 levels (approximately 10-fold after correction for loading using the 36B4 protected fragment for normalization) when antisense hsp27 expression was induced with Cd. Antisense transfectant Tf.10 exhibited a 4-fold decrease in hsp27 levels when antisense expression was induced by Cd.

Growth of the hsp27-transfected Cell Lines. The anchorage-dependent growth of the sense hsp27 Tf.9 construct and the antisense Tf.63 construct, as compared to their appropriate controls (vector-alone transfectants), is shown in Fig. 2A. Modulation of hsp27 expression affected the proliferation of both of these transfected lines. Growth was significantly increased with hsp27 overexpression in the sense Tf.9/MDA-MB-231 cells, ($P \leq 0.001$), and decreased in hsp27 antisense Tf.63/MCF-7/MG cells ($P \leq 0.001$).

To confirm the association between hsp27 expression and proliferation, we next analyzed the anchorage-independent growth of these two transfectants in soft agar (Fig. 2B). The hsp27 sense-transfected Tf.9 cells showed a 3.85-fold increase in anchorage-independent growth in soft agar as compared to control cells ($P \leq 0.01$). Besides

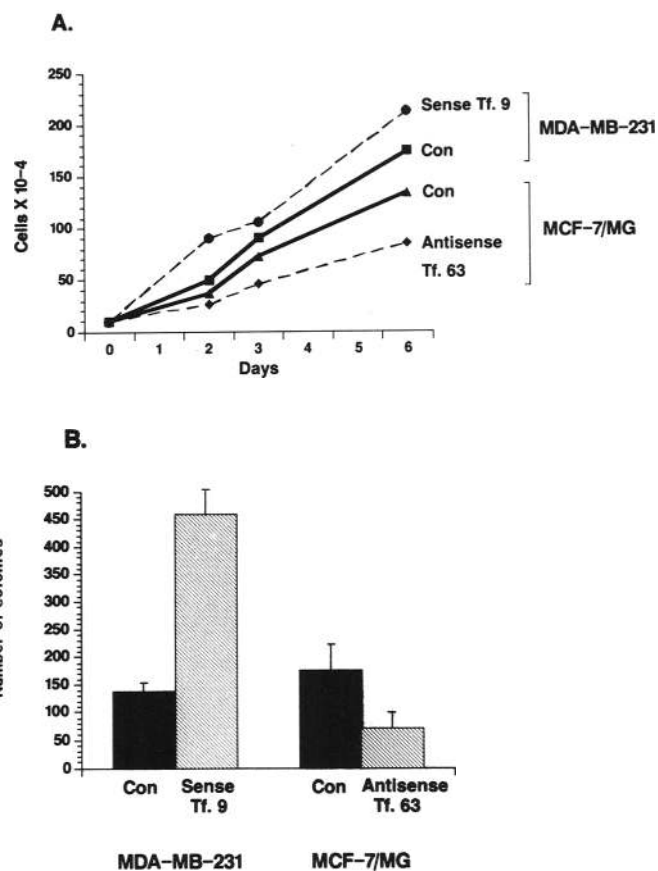


Fig. 2. Anchorage-dependent (A) and anchorage-independent (B) growth by hsp27 expression. Growth is shown for MDA-MB-231 cells Tf.9, transfected with hsp27 in sense direction, and MCF-7/MG cells Tf.63, transfected with hsp27 in antisense orientation, as compared to their appropriate controls. Only MCF-7/MG control and Tf.63 cells were exposed to cadmium chloride; cadmium chloride had no effect on MDA-MB-231 Tf.9 cells (see "Results").

this increase in the number of soft agar colonies, the growth effects of hsp27 overexpression were also quite obvious by microscopic evaluation; the Tf.9 colonies were very large and grew in multiple layers (Fig. 3A, Dox, $\times 2.5$). Conversely, the clonogenicity of the antisense Tf.63 cells in soft agar was less than one-half that of control cells ($P \leq 0.01$) (Fig. 2B). All experiments described were repeated with two different independent clones, Tf.6 (sense) and Tf. 10 (antisense), whose levels of hsp27 expression were shown in Fig. 1, A and B. Similar growth effects were seen in these clones (results not shown).

Drug Sensitivity of the hsp27 Transfectants. To analyze the effect of hsp27 expression on Dox resistance, we next determined the Dox sensitivity profiles of the transfected cells as determined by soft agar assays (Table 1). Two individual clones from both sense and antisense transfections are shown. The estimated Dox IC_{50} from control, vector-alone transfected MDA-MB-231 cells was 0.08–0.09 $\mu\text{g}/\text{ml}$, while the IC_{50} in hsp27-overexpressing MDA-MB-231 transfectants increased (0.18–0.38 $\mu\text{g}/\text{ml}$) For an estimate of fold change in resistance which is reflective of the general effect of hsp27 overexpression, we combined experimental data from both of these hsp27-overexpressing transfectants. A 3-fold increase in Dox resistance was seen concomitant with hsp27 overexpression; these results were statistically significant ($P \leq 0.001$). Furthermore, the morphology of these clones after Dox treatment was dramatically affected. In the presence of 0.1 $\mu\text{g}/\text{ml}$ of Dox, control cells only formed small, apparently sick colonies (Fig. 3A, b), whereas the sense hsp27 Tf.9 transfected cells formed larger, much more healthy appearing colonies (Fig. 3A, d; $\times 10$).

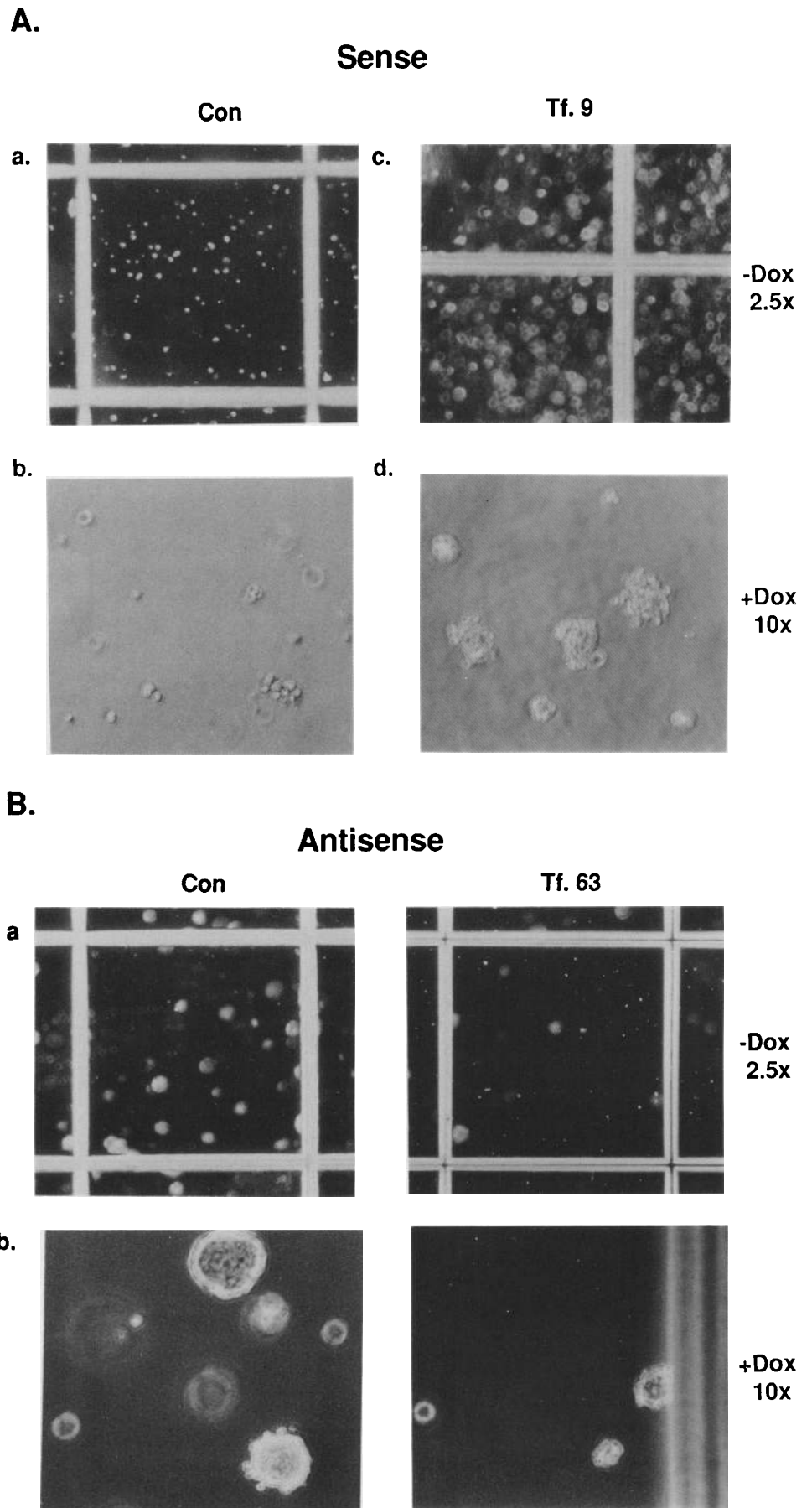


Fig. 3. Soft agar colony growth of MDA-MB-231 cells (A) and MCF-7/MG cells (B). Colonies were grown in the absence (a and c; $\times 2.5$) or presence of 0.1 $\mu\text{g/ml}$ Dox (b and d, $\times 10$ magnification); a and b represent control transfected cells; c and d represent hsp27-transfected cells.

Table 1 Effect of hsp27 expression on Dox resistance

Transfected cell line	Clone ^a	Control transfectant IC ₅₀ (range)	hsp27 transfectant IC ₅₀ (range)	Fold resistance/sensitivity ^b (95% CI)	Statistical significance ^c
MDA-MB-231 (sense)	Tf.9	0.09 (0.04–0.16)	0.18 (0.11–0.29)	3.16 resistant (2.68–3.73)	$p \leq 0.001$
	Tf.6	0.08 (0.08–0.08)	0.38 (0.31–0.045)		
MCF-7/MG (antisense)	Tf.63	0.33 (0.20–0.46)	0.06 (0.03–0.08)	2.8 sensitive (1.77–4.44)	$p \leq 0.012$
	Tf.10	0.36 (0.27–0.46)	0.25 (0.16–0.35)		

^a Data shown represents three individual experiments with Tf.9, two experiments with Tf.6, four experiments with Tf.63, and two experiments with Tf.10.

^b Fold changes were estimated by back-transforming means of differences in log transformed IC₅₀. CI, confidence interval.

^c Log transformed IC₅₀ for control and transfectant in a repeated measures analysis of variance were used for estimating statistical significance.

Consistent with these results, the IC₅₀ of MCF-7/MG cells decreased after transfection of hsp27 in the antisense orientation (from 0.33–0.36 to 0.25–0.06 $\mu\text{g/ml}$), corresponding to an overall 3-fold increase in Dox sensitivity which was statistically significant ($P \leq 0.012$). Furthermore, in the presence of Dox, the colonies were noticeably and reproducibly smaller. For instance, in the antisense Tf.63 cells (Fig. 3B, d) as compared to control cells (Fig. 3B, b), although this effect was not as overwhelming as the effect seen in sense hsp27-overexpressing MDA-MB-231 cells. The consistency of the results with two individual clones for the sense and antisense experiments support our conclusion that hsp27 overexpression affects both the growth and Dox sensitivity of human breast cancer cells.

Discussion

The role of the small heat shock protein hsp27 in Dox resistance and its involvement in regulation of cell growth were investigated in human breast cancer cell lines with different levels of hsp27 expression as a result of hsp27 cDNA transfection in the sense or antisense orientation. As we hypothesized, after transfection of hsp27 in the sense orientation into low endogenous hsp27-expressing MDA-MB-231 cell lines, an increase in hsp27 expression and an increase in Dox resistance were observed. In contrast, high hsp27-expressing MCF-7/MG cells showed an increase in Dox sensitivity after transfection with hsp27 in the antisense orientation which resulted in a marked reduction in expression. This strongly suggests that hsp27 is involved in Dox resistance in human breast cancer cells. Interestingly, the drug sensitivity of one antisense transfected MCF-7/MG cell line (Tf.63) (IC₅₀ 0.06 $\mu\text{g/ml}$), which were inherently Dox-resistant, was increased to that of the low-expressing parental MDA-MB-231 cells (IC₅₀ 0.08–0.09 $\mu\text{g/ml}$), after reduction of hsp27 levels by antisense expression. This suggests that manipulation of hsp27 levels, either genetically as we have demonstrated here, or perhaps pharmacologically, may be useful in reversing Dox resistance in breast cancer patients.

Since heat shock induces hsp27 accumulation, these results might explain in part our reported protective effect of prior heat shock treatment on Dox sensitivity of breast cancer cells (3). These results are also in general agreement with a previous report of increased drug resistance to several different classes of drugs after hsp27 sense transfection into CHO cells (14). However, our results differ in that hsp27 expression is specifically associated only with Dox resistance in human breast cancer cells.

At this time, however, the mechanisms underlying hsp-induced drug resistance are unclear. There are contradictory reports regarding the association between hsp-mediated drug resistance and reduced drug accumulation in different cell types. Huot *et al.* (14) did not find an increased efflux or decreased retention of daunorubicin (a Dox analogue) in hsp27-transfected CHO cells. However, if CHO cells are first exposed to Dox followed by a heat treatment, accumulation of

this drug was significantly reduced, primarily as a result of decreased passive drug diffusion rather than increased efflux in the heated cells (15). We have not yet performed these studies with our transfectants and thus cannot comment on changes in Dox uptake and efflux at this time.

The issue of Dox resistance is a very important one in clinical breast cancer since Dox is the most active single agent currently used in the adjuvant treatment of breast cancer. Two- to five-fold changes in Dox resistance are relatively low in comparison to the values reported for *in vitro* models of the *mdr* gene (16). However, chemotherapeutic drugs are normally used at or near maximally tolerated doses, and if our results hold up *in vivo*, two- to five-fold changes in the effectiveness of a fixed dose would be clinically important. Furthermore, other non-*mdr* mechanisms of drug resistance, such as mutation, increased levels of target enzymes (*e.g.*, dihydrofolate reductase) or increased efficiency of DNA repair, also show changes in resistance ranging 2–10-fold (17). Finally, in the clinic, drug resistance in this range is much more common than the large values seen in *mdr*-related model systems.

Finally, we have demonstrated for the first time a statistically significant, positive association between hsp27 expression and *in vitro* growth. This was not anticipated, since current dogma suggests that increased proliferation is associated with increased drug sensitivity. Furthermore, overexpression of the murine hsp25 in Ehrlich ascites tumor cells leads to a growth inhibition (6). However, the differences in our model system with that used by Knauf *et al.* (6), and a possible concentration-dependent influence of hsp27 on proliferation, may explain this apparent contradiction in results. Our analysis, however, does strongly support the hypothesis that hsp27 may be involved, not only in drug resistance, but also in the control of cell proliferation. Therefore, modulation of hsp27 levels may be a clinically useful potential target for reversing drug resistance and controlling breast tumor growth.

Acknowledgments

We thank Dr. Dwight Kaufman (National Cancer Institute, Bethesda, MD) for the gift of pMTP-5H vector, Lynn Corder for technical assistance, and Gladys Locos for typing the manuscript. The authors would also like to thank Dr. Gary Chamness for a critical evaluation of the manuscript.

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