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Divergent effects of hyperosmolality on stress-response (heat shock) protein expression in cultured human tumor cells: an immunocytochemical study

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Abstract. Exposing cells to adverse conditions usually elicits expression of stress-response (heat shock) proteins (srp). Here we show that hyperosmolar growth conditions do not uniformly affect srp expression in MCF-7 and HeLa S3 cells, derived from carcinoma of the breast and cervix, respectively. Thus, whereas srp 27 expression was increased in MCF-7, but not in HeLa S3, the opposite was the case with srp 72. On the other hand, hyperosmolality did not induce αB-crystallin or ubiquitin in either cell line. These findings show that srp expression by the human tumor cells studied is non-coordinate, suggesting that each srp is independently modulated.
Key words. Stress-response proteins; heat shock proteins; hyperosmolality; human tumor cells; MCF-7; HeLa S3; immunocytochemistry.

Since the discovery by Ritossa that small temperature shifts cause new puffs to appear in the polytene chromosomes of *Drosophila*,[1] it has become firmly established that all organisms exhibit essential, highly conserved, and exquisitely regulated cellular responses to adverse conditions[2]. These adaptive mechanisms involve the inducible regulation of specific sets of stress-response (heat shock) proteins (srp). In addition to temperature, a variety of other factors and apparently unrelated agents act as srp inducers[3]. Moreover, the proteins are also involved in cellular processes under non-stress conditions[2,4] and their expression seems to be constitutively or developmentally controlled[5].

Recent studies have shown that hyperosmotic stress induces increased srp expression in cultured canine kidney cells[6]. Although this observation seems relevant for the understanding of normal renal medullar function, it is not known whether the response is unique to kidney cells or is shared by other continuous cell lines, irrespective of their tissues or species of origin. Here we report on immunocytochemical investigations in which we examined the effect of hyperosmolality on srp 27, srp 72, αB-crystallin and ubiquitin in MCF-7 and HeLa S3 cells. These cell lines, derived from human carcinomas of the breast[7] and cervix[8], respectively, were chosen because mammary[9,10] and cervical tumors[11] are among the few human tumors [12-16] in which srp expression has been documented. Our results indicate that whereas hyperosmolality enhances the expression of srp 27, but not that of srp 72 in MCF-7, it elicits increased srp 72 expression in HeLa S3 cells without affecting srp 27. However, hyperosmolality did not induce αB-crystallin or ubiquitin in either cell line. These findings provide evidence that the effect of hyperosmolality on srp expression is not limited to cultured renal cells and document the non-coordinate response by the human tumor cells studied to hyperosmolar growth conditions.

Materials and methods

MCF-7 and HeLa S3 cells were routinely grown in plastic T80 flasks using Eagle's minimum essential medium supplemented with fetal bovine serum (10%), penicillin (100 units/ml), streptomycin (100 μg/ml) and amphotericin B (0.25 μg/ml). Medium was changed twice a week. A mixture of trypsin (0.05%)-EDTA (0.02%) was used for the weekly cell transfer[17]. The cultures were checked periodically for mycoplasmas and were found to be free of contamination. For the immunocytochemical analysis of srp expression, cells were grown in 8-compartment Lab-Tek glass chamber/slides[18,19] (Nunc, Inc., Naperville, Illinois, USA). Where indicated, the osmolality of the medium was increased by the addition of appropriate amounts of NaCl (from an autoclaved 3 M stock solution) 24 h after the transfer of cells into chamber/slides[17]. In some experiments the medium was rendered hyperosmolar by adding sorbitol (100 mM) or sucrose (120 mM)[18]. The osmolality of the medium was determined with a Model 3DII Advanced DigiMatic osmometer (Advanced Instruments, Needham Heights, Massachusetts, USA). Cells within the same slide to which no additions were made served as culture controls. After growth for 72 h at 37 °C in a humidified atmosphere of 5% CO_2 in air, the cell monolayers were fixed for 2.5 min with ice cold acetone:methanol (1:1), washed repeatedly with phosphate-buffered saline, pH 7.4 (PBS) and stored in PBS at 4 °C.

The following primary antibodies were used: an IgG_1 mouse monoclonal antibody (MAb) to srp 27 purified from MCF-7 cells (clone G3.1, high performance,

Ready-to-use, BioGenex, San Ramon, California, USA); an IgG_1 mouse MAb to purified srp 72/73 from HeLa cells which recognizes constitutive and inducible srp 72 [diluted 1:500 in bovine serum albumin (1%)-containing PBS (BSA-PBS), Amersham, Arlington Heights, Illinois, USA]; an affinity-purified rabbit antibody against αB-crystallin (diluted 1:500 in BSA-PBS, gift of Dr. J. E. Goldman, Columbia University), and an affinity-purified rabbit antibody to keyhole limpet hemocyanin-conjugated ubiquitin (diluted 1:1000 in BSA-PBS, gift of Dr. S.-H. Yen, Albert Einstein College of Medicine). The specificity and utility of the antibodies for the immunocytochemical demonstration of the respective epitopes in human material have been documented repeatedly[12,13,20]. The immunocytochemical assays with all four antibodies were performed concurrently on the same 8-compartment chamber/slide[18,19]. Immunostaining was done as previously described[18,20] using the avidin-biotin-complex (ABC) immunoperoxidase method[21]. Briefly after quenching the endogenous peroxidase with H_2O_2 and exposure to normal horse serum (blocking reagent), the fixed cells were incubated with the primary antibodies for 18 h at 4 °C. PBS was used in the assay controls. Visualization of bound antibodies was done with the respective Vectastain ABC kit (Vector Laboratories, Burlingame, California, USA) following the manufacturer's protocols; 3,3′-diaminobenzidine tetrahydrochloride (Dako, Glostrup, Denmark) was the final chromogen and methyl green was used as counterstain. The entire preparations were examined under high power (400×). No stained cells were seen in the control assays incubated with PBS.

Cells grown in T80 flasks to which excess NaCl was added 24 h after subculture were used for immunoblot analysis; as controls served cultures to which no additions were made. Cells were harvested as indicated above, washed with PBS, resuspended in HEPES-NaCl-KCl buffer (pH 7.9) and lysed. After centrifugation at $18000 \times g$ the cytosols (adjusted to similar protein concentrations) were used for immunoblotting, done according to the procedure of Towbin et al[22]. Appropriate molecular weight standards were included in the electrophoretic runs.

Results and discussion

Constitutive srp 27 expression was seen in MCF-7 cells grown at 37 °C in regular medium (290 mOsm/kg). The reaction product deposits displayed a granular pattern. Staining was restricted to the cytoplasm (fig. 1a); in no instance was nuclear staining seen. However, srp 27 expression in the control cultures was not uniform as there were cells that were less intensely stained than others or not stained at all. These observations reflect the known heterogeneity of tumor cell populations with respect to srp 27 expression[10,13]. By comparison, the staining intensity of MCF-7 cells grown in medium supplemented with additional NaCl (final osmolality: 490 mOsm/kg) was uniformly increased and there were no cells that failed to react with the anti-srp 27 MAb (fig. 1b). Similar results were obtained with cells grown in medium supplemented with sorbitol or sucrose, thus indicating that the observed effect was osmotic rather than ionic. On the other hand, growth in hypertonic medium had no significant overall effect on srp 72 expression in MCF-7 cells, as the staining intensities of control (fig. 1c) and experimental cultures (fig. 1d) were similar. The immunocytochemical results were corroborated by Western blotting (fig. 2). Staining of MCF-7 cells with the MAb to srp 72 was always restricted to the cytoplasm, the normal locale of the constitutive protein[23]. Evidence that hyperosmolality did indeed exert stress and affected cell proliferation was obtained from measuring total cell protein content after 72 h exposure[18]. Thus, whereas in control cultures the total protein content was 1.53 mg/flask, in the experimental cultures it was 0.67 mg/flask. The reduction in cell proliferation was accompanied by morphological modifications such as an increase in the size of the nucleus (fig. 1b).

The effects of hyperosmolality on srp 27 and srp 72 expression in HeLa S3 cells were the opposite of those seen with MCF-7. Thus, it had no effect on srp 27 as the staining intensity of HeLa S3 cells exposed to hyperosmolar conditions (390 mOsm/kg) was similar to that of cells grown in regular medium (fig. 1e and 1f). By contrast, the effect on srp 72 was striking. As shown in figure 1g, considerable heterogeneity was evident in the control cultures, with the cytoplasm of some cells more intensely stained than that of others. By comparison, HeLa S3 cells grown in hyperosmolar medium exhibited increased srp 72 expression, and reaction product deposits were detected not only in the cytoplasm, but also in the nucleus (fig. 1h). The observed increase in srp 72 corroborates at the protein level the finding that hyperosmotic shock augments specific mRNA levels in canine kidney cells[6], whereas the accompanying intracellular redistribution mimics that seen in animal cells subjected to heat and other stressing factors[23]. Moreover, the observed effect could reflect the ancient origin[24] of srp, since DnaK, the sole member of the srp 72 family in *E. coli* and 50% homologous with the human protein, also increases in response to osmotic stress.

The examination of MCF-7 and HeLa S3 for the newly recognized srp, αB-crystallin[26] revealed that these cells did not express the epitope recognized by the respective antibody as no reaction was seen with control cultures or with cells exposed to hyperosmolality. This observation is in accordance with reports that cultured human glioma cells lack αB-crystallin[27] and that heat shock increases expression of the closely homologous srp 27[28] without affecting αB-crystallin[29]. However, αB-crystallin was

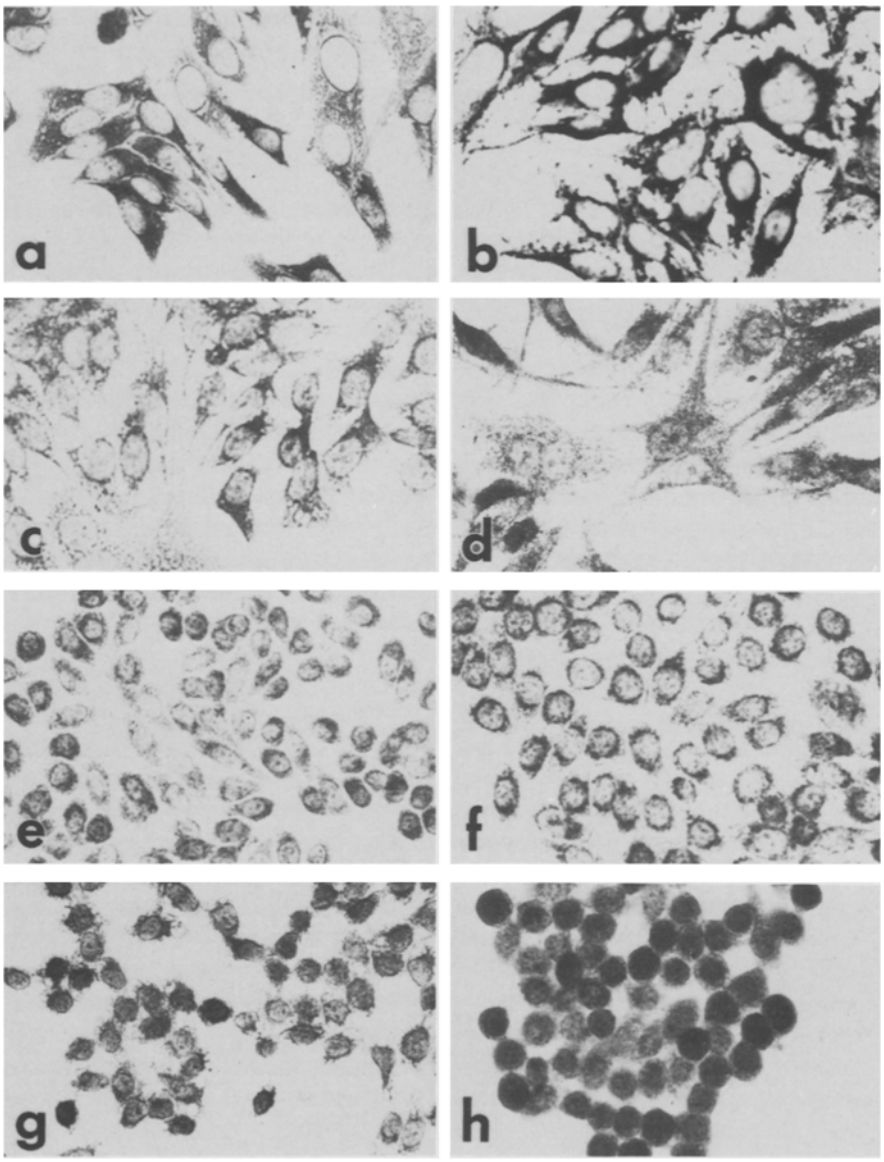

Figure 1. Immunocytochemical demonstration of the effect of hyperosmolality on srp 27 and srp 72 expression:
a srp 27 of control MCF-7 cells (290 mOsm/kg). *b* srp 27 of MCF-7 cells grown for 7 h in hyperosmolar medium (490 mOsm/kg).
c srp 72 of control MCF-7 cells. *d* srp 72 of MCF-7 cells grown for 72 h in hyperosmolar medium.
e srp 27 of control HeLa S3 cells. *f* srp 27 of HeLa S3 cells grown for 72 h in hyperosmolar medium (390 mOsm/kg).
g srp 72 of control HeLa S3 cells. *h* srp 72 of HeLa S3 cells grown for 72 h in hyperosmolar medium.
(Magnification: 400×)

identified and shown to be inducible by heat shock in a rat glioma line[27] and in a human ovarian tumor cell line[30], as well as by hypertonic stress (550–600 mOsm/kg) in primary cultures of dog lens epithelial cells and glomerular endothelial cells[31], but not in Chinese hamster ovary cells[31]. With respect to ubiquitin, an 8 kD srp[32], only nuclear immunostaining was observed in MCF-7 and HeLa S3 cells and its intensity was not increased when the cells were grown in hyperosmolar medium.

From our results it is evident that hyperosmolality exerts dissimilar effects on srp 27 and srp 72 of the tumor cells studied, pointing to the non-coordinate control of srp in eukaryotes[33]. On the other hand, the high degree of conservation of srp during evolution[2,24] suggests that the disparate effects of hyperosmolality on human cells conceivably reflect the very ancient roles that these proteins play in maintaining essential cellular functions under environmental conditions that endanger cell survival. Although the ultimate significance of our observations remains to be established, it is possible that they may have implications with respect to tumor growth and survival. It is of interest to note in this context the recent reports that virally or spontaneously transformed Balb/c 3T3 cells adapt poorly and much more slowly to hyperosmotic stress than their non-

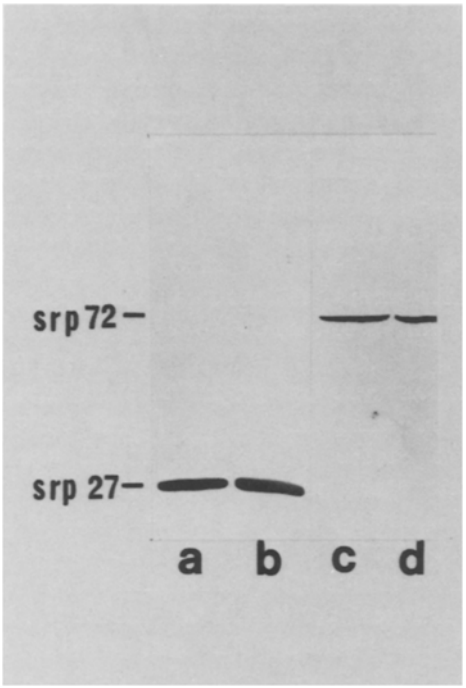

Figure 2. Immunoblots of srp 27 and srp 27 of control (*a*, *c*) and hyperosmolar (*b*, *d*) cell cultures.

transformed counterparts[34,35]. On the basis of these observations it was suggested that hyperosmotic treatment, instead of or in addition to hyperthermia, might be considered as a means of selectively affecting the survival of tumors cells[34].

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