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ORIGINAL ARTICLE

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Inhibition of proliferation and induction of apoptosis by abrogation of heat-shock protein (HSP) 70 expression in tumor cells

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Abstract Tumor cells often express elevated levels of heat-shock protein (HSP) 70. The present study was designed to invesitgate the role of HSP70 in the proliferation and survival of tumor cells in the human system. When Molt-4 and other tumor cells were treated in vitro with HSP70 antisense oligomer, they displayed propidium-iodide-stained condensed nuclei (intact or fragmented). A ladder-like pattern of DNA fragments was observed with HSP70 antisense-oligomer-treated tumor cells in agrose gel electrophoresis, which was consistent with internucleosomal DNA fragmentation. Flow cytometry analysis revealed the hypodiploid DNA peak of propidium-iodide-stained nuclei in the antisense-oligomer-treated cells. The apoptosis induced by HSP antisense oligomer was dose- and time-dependent. The antisense oligomer induced apoptosis mainly in tumor cells at G1 and S phase, resulting in an inhibition of cell proliferation. HSP70 antisense oligomer caused DNA-sequence-specific inhibition of HSP70 expression, which preceded apparent apoptosis. These results indicate that HSP70 antisense treatment inhibits the expression of HSP70, which in turn inhibits cell proliferation and induces apoptosis in tumor cells and suggest that HSP70 is required for tumor cells to proliferate and survive under normal condition.

Key words Heat-shock protein · Tumor cells · Apoptosis Proliferation

Introduction

One major aspect of heat shock and other stress responses is the rapid and exclusive synthesis of highly conserved proteins, termed heat-shock proteins (HSP) or stress proteins [4, 9, 11–13, 16, 22, 23, 24]. Among HSP families HSP70 is known to protect mammalian cells from stress-induced damages [4, 9, 11–13, 16, 23, 24]. Of interest is the recent finding that HSP70 is prerequisite as a molecular chaperone for the maturation of proteins in cells growing under normal conditions [9, 23]. It has been reported that tumor cells, especially malignant tumor cells express elevated levels of HSP70 [12, 24, 8, 19, 22]. The increased expression of HSP70 was associated with the progression of human cancer [19]. In addition, HSP70 was shown to be associated with mutant P53. P53 is a nuclear phosphoprotein, which is normally expressed at low levels and plays an important role in the regulation of cell growth [19]. These studies did not, however, clarify whether the increased expression of HSP70 is required for the proliferation and survival of tumor cells.

Since HSP70 plays an important role as a molecular chaperone in various aspects of protein maturation, it is conceivable that the specific inhibition of its expression may affect the proliferation and survival of tumor cells under normal growth conditions (normal temperature). The present study was designed to confirm the above suggestion by inhibiting HSP70 expression wth HSP 70 antisense oligodeoxynucleotides in human tumor cells.

Materials and methods

Synthesis and purification of oligomers

Nuclease-resistant phosphorothioate oligodeoxynucleotides (antisense, sense and nonsense) were synthesized automatically with a 381 DNA synthesizer and purified by high-pressure liquid chromatography and reverse-phase chromatography as described [1, 18]. HSP70 antisense oligomer (5′-CGCGGCTTTGGCCAT-3′) was complementary to the initiation codon and four downstream codons of human HSP70 mRNA [12]. The corresponding sense oligomer (5′-ATGGCCAAAGCCGCG-3′) and nonsense oligomer (5′-CGGGTATGCTTCGCC-3′) were used as controls. These oligomers were taken up by Molt-4 within 90–180 min and remained intact for over 24 h in the cells. The specific inhibition of HSP70 expression by the antisense oligomer was analyzed by Western blot, quantitative immunofluorescence and Northern blot methods.

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Antisense treatment

Exponentially growing Molt-4 cells at 2 X 10^5/ml in RPMI-1640 medium containing 10% fetal bovine serum and antibiotics were exposed to various doses of HSP antisense oligomers or sense or nonsense oligomers for varyious lengths of time. The culture medium was replaced every 24 h by fresh medium containing the same concentration of HSP70 antisense or sense or nonsense oligomers. Control culture was left untreated.

Morphological analysis

The number of viable cells was determined by a trypan blue dye exclusion test, and the percentage inhibition was calculated by the following formula, as described: inhibition % = $[(N-N_T)/(N-N_0)] \times 100$, where N is the number of untreated cells cultured for n days, N_0 is the cell number on day 0, and N_T is the number of treated cells cultured for n days [1]. For the identification of apoptotic cells, cells were resuspended in hypotonic propidium iodide solution containing 50 μg propidium iodide/ml in 0.1% sodium citrate plus 0.1% Triton X-100 and examined by fluorescence microscopy [22].

Agarose gel DNA electrophoresis

The pattern of DNA cleavage was analyzed by agarose gel electrophoresis as described [3, 10, 22]. Briefly, cells (3×10^6) were lysed with 0.5 ml lysis buffer containing 5 mM. TRIS/HCl, pH 8, 0.25% Nonidet P-40 and 1 mM EDTA; added by RNAase A (Sigma) was added at a final concentration of 200 μg/ml and incubated for 1 h at 37 °C. The cells were then treated with 300 μg proteinase K/ml for an additional hour at 37 °C. After addition of 4 μl loading buffer, 20-μl samples in each lane were subjected to electrophoresis on 1.5% agarose at 50 V for 3 h. DNA was stained with ethidium bromide.

Flow cytometry

Apoptotic cells were also identified by flow-cytometric analysis after propidium iodide staining in hypotonic buffer as described previously [17, 22]. Briefly, cells were suspended in 1 ml hypotonic fluorochrome solution containing 50 μg propidium iodide/ml in 0.1% sodium citrate plus 0.1% Triton X-100 (Sigma), and the cells were analyzed by the use of a FACScan cell sorter (Becton Dickinson, Mountain View, Calif.) with Cell Fit software. Apoptotic cells appeared in the cell-cycle distribution as cells with DNA content of less than that of G1 cells. Cell-cycle analysis was also performed simultaneously.

The level of intracellular HSP was quantitatively measured by flow cytometry, as described elsewhere [8, 18]. Briefly, cells were fixed with paraformaldehyde, permeabilized with Triton X-100 and washed. Monoclonal antibodies (mAb) used as the first antibody were anti-HSP25 mAb (clone IAP-9; Sigma), anti-(heat-inducible HSP70) mAb (Amersham, Buckinghamshir, UK) and anti-HSP90 mAb (Funakoshi, Tokyo, Japan).

Western blot analysis

Western blot analysis was performed as described previously [22]. Briefly, 1×10^7 cells were lysed in 1 ml lysis buffer, and the protein concentration was determined by the bicinchoninic acid protein assay reagent. The samples were denatured in sample buffer, sodium dodecyl sul and the proteins were separated by SDS/PAGE. Gels were electroblotted with Sartoblot onto a polyvinylidene difluoride membrane. The membrane blots were rinsed with 20 mM Tris, 500 mM NaCl. 0.05% Tween-20, (pH 7.5) and blocked by 3% gelatin. The blots were incubated first with anti-HSP70 mAb and then with a biotinylated second antibody, and were then transferred to Vectastain ABC. 3.3′-Diaminobenzidine substrate kits for horseradish peroxidase (Vector Laboratories) were used for development of color. Total cellular protein from 2×10^5 viable cells was added in each lane.

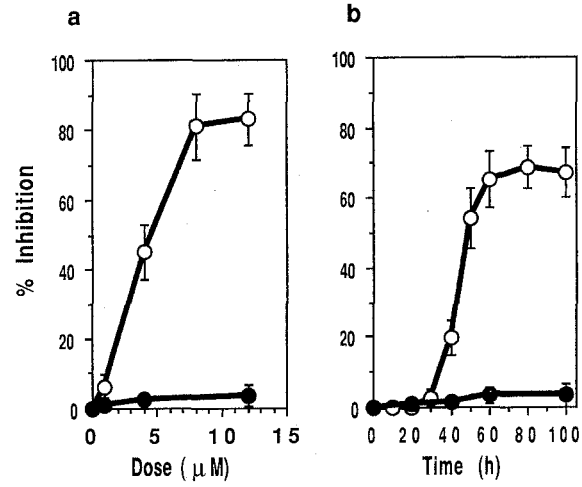


Fig. 1a, b Dose- (**a**) and time- (**b**) dependent inhibition of proliferation by HSP70 antisense oligodeoxynucleotides. Molt-4 cells were treated with various doses of HSP70 antisense oligomers (○) or sense oligomers (●) for 48 h (**a**), or they were treated with 5 μM antisense oligomers or sense oligomers for various times (**b**). Results are expressed as the means ± SD of three independent experiments

Results

Inhibition of cell proliferation by HSP70 antisense treatment

We have recently shown that Molt-4 cells expressed increased levels of HSP70. In an attempt to investigate whether the elevated HSP expression is prerequisite for the maintenance of tumor cell proliferation. Molt-4 cells were treated with HSP antisense oligomers. The antisense treatment resulted in an inhibition of proliferation of Molt-4. The antisense effect was dependent on the dose of antisense oligomers and time of incubation, being observed at 1 μM and reaching a maximum at 12 μM for 48 h (Fig. 1a, b). The antisense treatment not only inhibited cell proliferation, but also caused cell death when determined by the trypan blue dye exclusion test. By contrast, treatment with sense oligomers or nonsense oligomers had no effect. These results indicate that HSP70 antisense treatment of tumor cells inhibits cell proliferation but also induces cell death.

Induction of apoptosis by HSP antisense oligomer

In the next set of experiments we tried to identify the type of cell death induced by HSP70 antisense oligomers. Fluorescence microscopic examination of propidium-iodide-stained cells demonstrated the features of apoptotic cells, with a bright-red fluorescent condensed nuclei (intact or fragmented) in antisense-oligomer-treated cells, By contrast, the sense- or nonsense-oligomer-treated cells showed red, diffusely stained intact nuclei, the features of which were identical to those of untreated cells (Fig. 2a). Furthermore, agarose gel electrophoresis analysis revealed

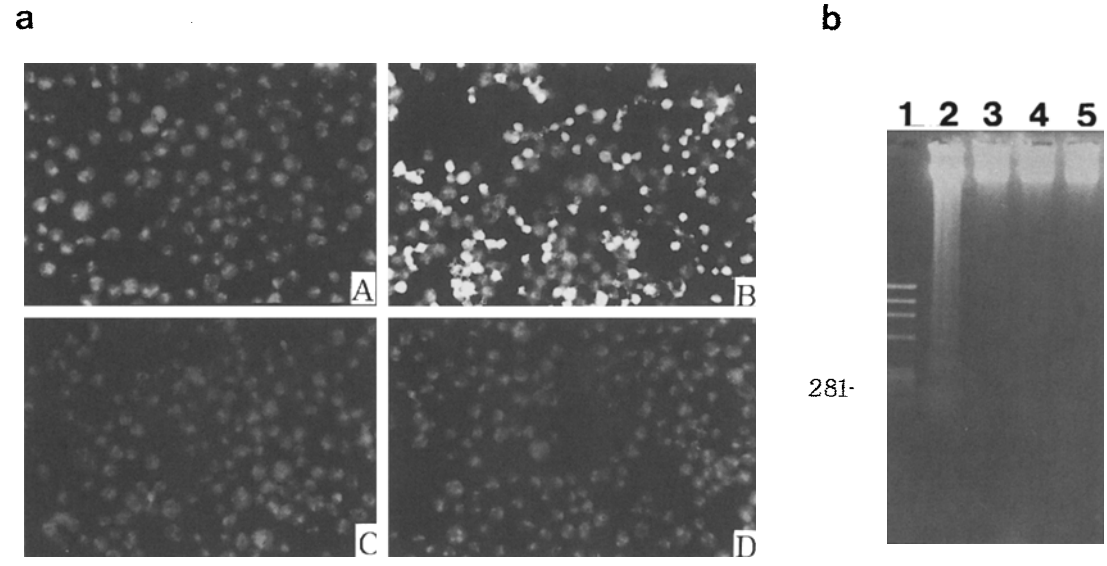


Fig. 2a, b Induction of apoptosis by HSP70 antisense oligomers. Molt-4 cells were treated with 8 μM HSP70 antisense oligmers, HSP70 sense oligomers or HSP70 nonsense oligomers for 96 h. **a** Fluorescence-microscopic appearance of propidium-iodine-stained nuclei of Molt-4. *A* Untreated, *B* antisense oligomer, *C* sense oligomer, *D* nonsense oligomer. **b** Agarose gel electrophoretic pattern of DNA. *Lane 1* marker, *lane 2* antisense treatment, *lane 3* sense treatment, *lane 4* nonsense treatment, *lane 5* untreated

a ladder-like pattern of DNA fragments consisting of about 180–200 base pairs in HSP-antisense-oligomer-treated cells, consistent with internucleosomal DNA fragmentation (Fig. 2b). The apoptosis-inducing activity of HSP70 antisense oligomer was not confined to Molt-4 cells but was also observed with other tumor cells, including the K562 erythroid leukemia, Raji Burkit lymphoma and MACS ovarian carcinoma cell lines, though the susceptibility to HSP70 antisense oligomer differed among cell lines (data not shown). By contrast, the same 3-day treatment with 8 μM antisense oligomer of normal and phytohemagglutinin-activated T lymphocytes resulted in no change in the porportion of apoptotic cells quantified by flow cytometry, which was 4.3 ± 2.3% (untreated, 3.9 ± 3.4%) and 5.6 ± 3.2% (untreated, 5.2 ± 2.7%) respectively. It is thus evident that HSP antisense oligomer treatment induces apoptosis in tumor cells but not in normal cell types.

Kinetics of induction of apoptosis and analysis of cell cycle

Kinetic studies revealed that the apoptosis induced by HSP70 antisense oligomers was dependent on their doses, being observed at 1 μM and reaching a maximum at 12 μM when analysed by flow cytometry (Fig. 3a, b). The increased number of apoptotic cells was detected after 30-h of continuous antisense treatment, reaching a maximum by 60 h (Fig. 4b). Next, a cell cycle affected by antisense oligomers was analyzed by flow cytometry. The number of G1- and S-phase cells among the total cells (hypodiploid cells and cells with diploid DNA) decreased when the number of hypodiploid/apoptotic cells increased as a result of an elevation of antisense oligomer dose and prolongation of incubation time (Fig. 4a, b). Treatment with lower doses of antisense oligomers induced apoptotic cells especially at S-phase. It thus seems likely that the inhibition of cell proliferation by HSP70 antisense oligomers may in part be a result of apoptotic cell death.

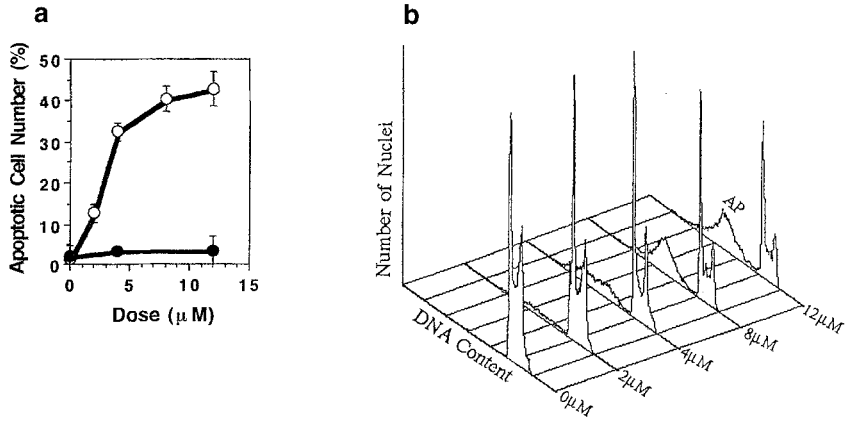


Fig. 3a, b Kinetics of induction of apoptosis by HSP70 antisense treatment. **a** The dose/response curve of antisense-induced apoptosis in DNA fluorescence histogram. Molt-4 cells were treated with 1–12 μM antisense (○) or sense oligomer (●) for 72 h. Results are expressed as the means ± SD of two independent experiments. **b** Corresponding representative DNA fluorescence histograms of Fluorescence 2-height (FL2-H); AP, apoptosis

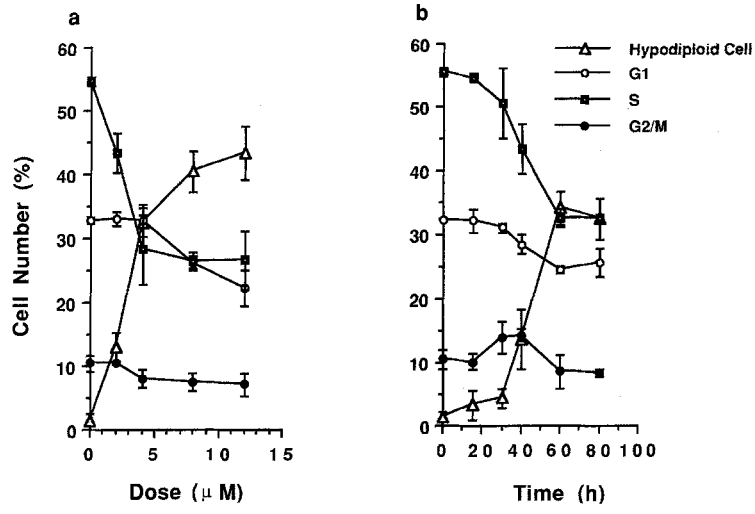
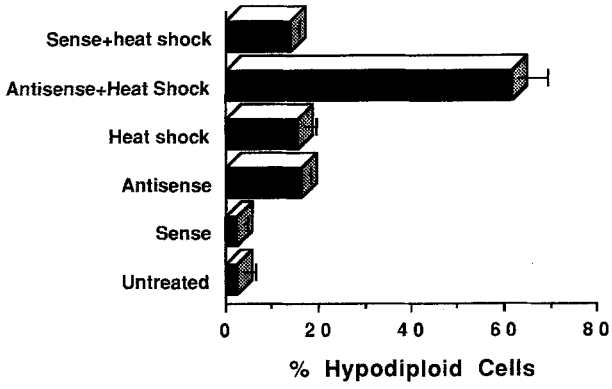


Fig. 4a, b Cell-cycle specificity of HSP70-antisense-oligomer-induced apoptosis. Molt-4 cells were treated with various doses of HSP70 antisense oligomers for 72 h (**a**), or with 5 μM HSP70 antisense oligomers for various times (**b**). Hypodiploid/apoptotic cells were calculated by DNA fluorescence histograms of FL2-H. Results are expressed as the means ± SD of two independent experiments

Enhancement of heat-shock-induced apoptosis by HSP70 antisense oligomer treatment

Since HSP70 played an important role in protecting cells from injury or repairing damage imposed by heat shock, and since heat shock itself induced apoptosis, we further investigated whether the inhibition of HSP70 by antisense treatment enhanced the induction of apoptosis by heat shock. To this end Molt-4 cells were treated with HSP70 antisense oligomers for 24 h, then exposed to heat shock at 42 °C for 2 h and subsequently cultured at 37 °C for an additional 24 h in the presence of the antisense oligomer. The number of apoptotic cells induced by heat shock was significantly higher in the antisense-oligomer-treated cells than in untreated cells (Fig. 5). Taken together, HSP70 antisense treatment induced apoptosis not only in normally growing tumor cells but also in heat-stressed cells.

Fig. 5 Effects of HSP70 antisense oligomers on heat-shock-induced apoptosis. Molt-4 cells were first treated with 8 μM antisense or sense oligomers for 24 h, exposed to heat shock at 42 °C, for 2 h, and then maintained at 37 °C for an additional 24 h in the presence of HSP70 antisense or sense oligomers. Also, Molt-4 cells were treated in parallel with antisense or sense oligomers or heat shock alone. Hypodiploid/apoptotic cells were calculated by DNA fluorescence histograms. Results are expressed as the means ± SD of duplicate samples



Specific inhibition of HSP70 by HSP antisense oligomers

Finally, Western blot and quantitative immunofluorescence analyses were performed to confirm the specific inhibition of HSP70 expression by HSP70 antisense oligomers. Treatment with HSP70 antisense oligomer of Molt-4 cells resulted in a dose- and time-dependent reduction of HSP70 expression, as demonstrated by Western blot analysis. By contrast, treatment with HSP70 sense oligomer had no effect (Fig. 6a). The specificity of antisense and sense treatment was confirmed by a quantitative immunofluorescence assay that measured relative levels of HSP70 and control proteins. HSP 70 antisense treatment resulted in a specific dose-dependent reduction in the level of HSP70 (Fig. 6b) without altering the level of HSP90 and HSP25.

Discussion

In the present study several observations have been made concerning the role of HSP70 in tumor cell proliferation and apoptosis. Tumor cells treated with HSP70 antisense oligomers displayed propidium-iodide-stained condensed nuclei, a ladder-like pattern of DNA fragments consistent with internucleosomal DNA fragmentation, and a hypodiploid DNA peak of propidium-iodide-stained nuclei analyzed by flow cytometry, which is consistent with the characteristics of apoptosis induced by other agents [2, 3, 10, 20–22]. The HSP70 antisense treatment affected cells at G1 and S phases and inhibited cell proliferation. DNA-sequence-specific inhibition of HSP70 expression was observed with the antisense-treated cells, which preceded apoptosis. The induction of apoptosis by heat shock was also enhanced by HSP70 antisense treatment.It seems likely, therefore, that HSP70 antisense treatment of tumor cells inhibits the expression of HSP70, which in turn induces apoptosis and inhibits cell proliferation. The inhibition of cell proliferation observed may in part be a result of cell death induced by apoptosis.

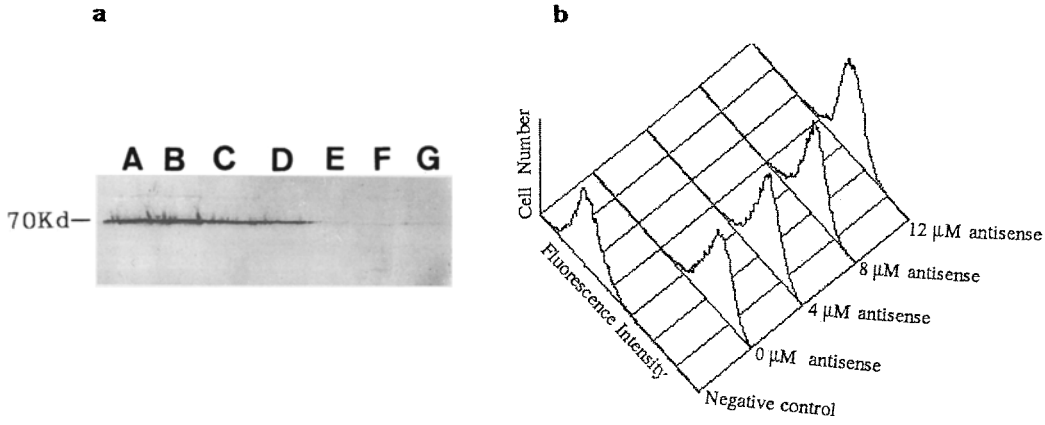


Fig. 6a, b Inhibition of HSP70 expression by HSP70 antisense oligomers. Molt-4 cells were treated with 8 μM antisense oligomers for various times or with various doses of HSP70 antisense oligomers for 24 h (**b**), and were tested for HSP70 expression by Western blot analysis (**a**) or by fluorescence-activated cell sorting (**b**). *Lane A* untreated, *lanes B, C, D, E, F, G* treatment with 8 μM antisense oligomer for 5, 10, 20, 30, 40, 60 h respectively

The present findings also suggest that HSP70 is essential for tumor cells to proliferate and survive under normal conditions. In this respect HSP70 has been shown to play an important role in normal cellular processes, including protein folding, assembly, disassembly and degradation [9, 16, 23], and in protection from environmental stress [4, 9, 11–13, 16, 23, 24]. It thus seems likely that the reduction of HSP70 levels by HSP70 antisense oligomer treatment renders tumor cells unable to maintain normal metabolism, resulting in an inhibition of proliferation and cell death. The importance of HSP70 in bacteria has been indicated by previous findings that *E. coli* with mutations in *dnaK* (*E. coli* 70-kDa HSP) has an altered cellular metabolism, including impaired DNA and RNA syntheses and compromised cell division [23]. In addition, substitution of wild-type alleles of yeast HSP70 genes with mutated genes constructed in vitro has resulted in abrogation of growth in yeast [6].

The biological significance of apoptosis is now generally accepted, though its mechanism is as yet poorly understood [5, 14, 15, 25]. Recently, considerable attention has been paid to the genetic control of apoptosis [5, 14, 15, 25]. Genes mediating or modulatin g apoptosis include P53, c-*myc*, *Fas/Apo-1*, *ras*, *bcl*-2, and *bax* [5, 14, 15, 25]. Our present results suggest that HSP70 also plays an important role in cell proliferation and apoptosis in tumor cells under normal growth condition. Thus, considerable emphasis should be placed on strategies that inhibit HSP70 expression in human tumor cells. Such approaches may provide a new focus for cancer treatment.

References

1. Agrawal S, Ikeuchi T, Sun D, Sarin PS, Konopka A, Maizel J, Zamecnik PC (1989) Inhibition of human immunodeficiency virus in early infected and chronically infected cells by antisense oligodeoxynucleotides and their phosphorothioate analogues. Proc Natl Acad Sci USA 86:7790
2. Barry MA, Behnke CA, Eastman A (1990) Activation of programmed cell death (apoptosis) by cisplatin, other anticancer drugs, toxins and hyperthermia. Biochem Pharmacol 40:2353
3. Barry MA, Reynolds JE, Eastman A (1993) Etoposide-induced apoptosis in human HL-60 cells is associated with intracellular acidification. Cancer Res 53:2349
4. Carper SW, Duffy JJ, Gerner EW (1987) Heat shock proteins in thermotolerance and other cellular processes. Cancer Res 47:5249
5. Clarke AR, Purdie CA, Harrison DJ, Morris RG, Bird CC, Hooper ML, Wyllie AH (1993) Thymocyte apoptosis induced by p53-dependent and independent pathways. Nature 362:849
6. Craig EA, Kramer J, Kosic-Smithers J (1987) SSC1, a member of the 70-kDa heat shock protein multigene family of *Saccharomyces cerevisiae*, is essential for growth. Proc Natl Acad Sci USA 80:4156
7. Eastman A (1990) Activation of programmed cell death by anticancer agents: cisplatin as a model system. Cancer Cells (Cold Spring Harbor) 2:275
8. Ferrarini M, Pupa SM, Zocchi MR, Rugarli C, Menard S (1994) Distribution of HSP72 and monomeric laminin receptor expression in human lung cancers infiltrated by g/d T lymophocytes. Int J Cancer 57:486
9. Gething M-J, Sambrook J (1992) Protein folding in the cell. Nature 355:33
10. Gorczyca W, Gong J, Ardelt B, Traganos F, Darzynkiewicz Z (1993) The cell cycle related difference in susceptibility of HL-60 cells to apoptosis induced by various antitumor agents. Cancer Res53:3186
11. Hosokawa N, Hirayoshi K, Kudo H, Takechi H, Aoike A, Kawai K, Nagata K (1992) Inhibition of the activation of heat shock factor in vivo and in vitro by flavonoids. Mol Cell Biol 12:3490
12. Hunt C, Morimoto RI (1985) Conserved features of eukaryotic hsp70 genes revealed by comparison with the nucleotide sequence of human hsp70. Proc Natl Acad Sci USA 82:6455
13. Jäätelä M and Wissing D (1993) Heat shock proteins protect cells from monocyte cytotoxicity: possible mechanism of self protection. J Exp Med 177:231
14. Lane DP (1993) A death in the life of P53. Nature 362:786
15. Lowe SW, Schmitt EM, Smith SW, Osborne BA, Jacks T (1993) P53 is required for radiation-induced apoptosis in mouse thymocytes. Nature 362:847
16. Morimoto RI (1993) Cells in stress: transcriptional activation of heat shock genes. Sciences 259:1409
17. Nicoletii I, Migliorati G, Pagliacci MC, Grignani F, Riccardi C (1991) A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J Immunol Methods 139:271

18. Reed J C, Stein C, Subasinghe C, Halder S, Croce CM, Yum S, Cohen J (1990) Antisense-mediated inhibition of BCL2 protooncogene expression and leukemic cell growth and survival: comparisons of phosphodiester and phosphorothioate oligodeoxynucleotides. Cancer Res 50:6565
19. Takahashi S, Mikami T, Watanabe Y, Okazaki M, Okazaki Y, Okazaki A, Sato T, Asaishi K Hirata K, Narimatsu E, Mori M, Sato N, Kikuchi K (1994) Correlation of heat shock protein 70 expression with estrogen receptor levels in invasive human breast cancer. Am J Clin Pathol 101:519
20. Uchida A and Fukata H (1993) Natural killer cell-derived granule protein: DNA fragmentation and apoptosis induced by framentin. Clin Immunol 25:646
21. Walton MI, Whysong D, O'Connor PM, Hockenbery D, Korsmeyer SJ, Kohn KW (1993) Constitutive expression of human Bcl-2 modulates nitrogen mustard and camptothecin induced apoptosis. Cancer Res 53:1853
22. Wei Y-Q, Zhao X, Kariya Y, Fukata H, Teshigawara K, Uchida A (1994) Induction of apoptosis by quercetin: involvement of heat shock protein. Cancer Res 54:4952
23. Welch WJ (1992) Mammalian stress response: cell physiology, structure/function of stress proteins, and implications for medicine and diseases. Physiol Rev 72:1063
24. Welch WJ, Suhan JP (1986) Cellular and biochemical events in mammalian cells during and after recovery from physiological stress. J Cell Biol 103:2035
25. Williams GT, Smith CA (1993) Molecular regulation of apoptosis: genetic control on cell death. Cell 54:777