Expression of HSP-28 and Three HSP-70 Genes during the Development and Decay of Thermotolerance in Leukemic and Nonleukemic Human Tumors

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ABSTRACT

Leukemic cells appear to develop less thermotolerance and then to lose their thermotolerance more rapidly than do other tumor cell lines. The reason for this phenomenon is not known. After heat shock (or other environmental stresses), mammalian cells preferentially synthesize a set of proteins known as heat shock proteins (HSPs). HSP-28 and the various isoforms of HSP-70 have been suggested as being responsible for the development of thermotolerance. In these studies, we have attempted to determine by their expression which HSPs positively correlate with the development and decay of thermotolerance and whether the expression of these genes could explain the differing thermotolerance response observed between leukemic and nonleukemic tumor cells. Polymerase chain reaction was used to detect the expression of HSP-28 and several HSP-70 genes. Our data indicate that the expression of all three heat-inducible HSP-70 genes, 70A (Hunt and Morimoto, Proc. Natl. Acad. Sci. USA, 82: 6455-6459, 1985), 70B (Voelmy et al., Proc. Natl. Acad. Sci. USA, 82: 4949-4953, 1985), and 70B' (Leung et al., Biochem J., 267: 125-132, 1990) correlate with the development and decay of thermotolerance in nonleukemic tumor cell lines after heat or arsenite treatment. HSP-28 (Hickey et al., Nucleic Acids Res., 4: 4127-4145, 1986) failed to correlate with thermotolerance development; it was not induced after 45°C primary heat shock. In leukemic cells, however, none of the HSPs were induced for extended periods of time. The lack of coordinate expression of HSP genes in cells of myeloid origin may explain the poor induction and maintenance of thermotolerance that is observed in these cells.

INTRODUCTION

When mammalian cells are exposed to mild heat exposure (or to other stresses, e.g., sodium arsenite), two events occur. Surviving cells become more resistant to a subsequent thermal exposure (thermotolerance), and these resistant cells synthesize (either preferentially or de novo) a set of proteins termed HSPs (1-5). It has frequently been suggested that these two events are causally related. Recent evidence has shown that transfection of human HSP-70 or HSP-28 genes into rat or Chinese hamster ovary cells, respectively, renders these cells resistant to subsequent heat damage (6, 7). Furthermore, the level of expression of these genes correlates with the degree of heat resistance in these transfectants.

There is one known difference between the responses of tumor cells of myeloid origin (leukemic cells) and other tumor cell types. Myeloid cells develop only a modest amount of thermotolerance and lose their thermotolerance more rapidly (8-10). We have initiated a study to determine whether this difference can be attributed to activation of genes of the HSP-70 family or HSP-28. As part of this study we have asked whether the expression of any specific isoform of HSP-70 or HSP-28 correlates with the development and decay of thermotolerance. The answer may assist in the development of assays for estimating thermotolerance in patients being treated with hyperthermia.

MATERIALS AND METHODS

Cell Culture and Maintenance. HL-60 (11) and KG-1 (12) cells are acute myelogenous leukemic cells. HT1080 and A549 are fibrosarcoma and lung adenocarcinoma cells, respectively. All cells were obtained from the American Type Culture Collection. The leukemic cells were maintained in Iscove's modified Dulbecco's medium plus 20% fetal calf serum and antibiotics (10). The solid tumor cells were maintained in minimal essential medium plus 10% fetal calf serum.

Cell Survival Assays. The leukemic cells were grown as described previously (10). Briefly, after appropriate treatments, cells were plated in 35-mm Petri dishes. The growth medium contained α minimal essential medium, 10% fetal calf serum, and 0.3% noble agar. KG-1 cells were supplemented with 150 units/ml of recombinant granulocyte-macrophage colony-stimulating factor (a gift of the Genetics Institute of Boston (13)). Plating efficiencies for HL-60 and KG-1 cells were 10-20% and 5-15%, respectively. HT1080 and A549 were grown in 60-mm dishes. After appropriate treatments they were trypsinized and plated for colony formation. All cell lines were incubated at 37°C, 98% humidity, and 5% CO₂ for 10-14 days. Only colonies with ≥50 cells were counted.

Synthetic Oligonucleotides. Oligonucleotides were synthesized with an ABI automated DNA synthesizer in the DNA synthesis facility at Stanford University. The sequences of the HSP-70A (14) and HSP-70B (15) genes chosen for amplification were from the 5' end untranslated regions. The sequences chosen for the HSP-70B' (16) gene were from the 3' end untranslated region. These sequences were chosen to eliminate possible cross-reactions with any other HSP-70 gene family members, since the divergence between genes increases in the 5' end and 3' end regions. The sequences chosen for the HSP-28 and actin genes were chosen from the coding sequence in the second and third exons. For HSP-28, the sequence of primer 2 was chosen in the region where the gene differs maximally from its pseudogene (17). The primers and probes for all genes are detailed in Table 1.

RNA and DNA Amplification (PCR) Assay. Total RNA was isolated by the guanidinium/CaCl₂ method as previously described (18). This procedure plus routine DNase treatment of each sample proved to yield minimal DNA contamination (19). Total RNA was quantitated by A₂₆₀ and electrophoresis in 6% formaldehyde/1% agarose gels. One μg of total RNA from control or treated cells was incubated in 1× amplification buffer (50 mM KCl, 10 mM Tris-Cl, pH 8.3), 1.5 mM Mg²⁺, and 0.01% gelatin together with 50 pmol of each of the priming nucleotides and 20 μM dNTP (ATP, GTP, CTP, and UTP) to a final volume of 50 μl. The Mg²⁺ concentration was determined to be 1.5 mM for all genes. Samples were placed in a thermal cycler (Ericomp Corp.), heated to 95°C for 5 min, cooled to 42°C for 2 min, at which time 2 units of AMV reverse transcriptase and 2.5 units of Taq polymerase (Perkin-Elmer-Citus) were added, and then incubated at 42°C for 5 min. The cycles of heating (95°C for 2 min), annealing (42°C for 2 min for all except HSP-28 and HSP-70B genes, where annealing was 55°C for 2 min), and polymerization (72°C for 2 min) were continued for 22 cycles (30 cycles for HSP-28 and HSP-70B, due to their lower amounts of mRNA). Twenty-two (or 30) rounds of amplification resulted in a linear decline in PCR products as the amount of mRNA added to the PCR reaction was decreased for all genes (19). Additionally, control experiments lacking reverse transcriptase were performed to check for DNA contamination (19).
Agarose Gel Electrophoresis, Blotting, and Hybridization. After PCR amplification, samples were electrophoresed in 1.8% agarose gels, vacuum blotted on Zeta probe nylon membranes (Biorad), and hybridized with the $\text{[^{32}P]y-ATP-labeled probe specific to each amplified product}$ as previously described (19, 20). The $\text{[^32}P]-labeled Southern blots were analyzed by 2-dimensional gel electrophoresis as previously described (21). Following electrophoresis, gels were stained with Coomassie blue R250 in 3.5% perchloric acid, destained in 7% acetic acid, enhanced, dried, and autoradiographed on Kodak SB-5 X-ray film at $-70^\circ\text{C}$.

RESULTS

Kinetics of Expression of $70A$, $70B$, $70B'$ and $HSP-28$ Genes after Heat Shock in Nonleukemic Tumor Cells during the Development and Decay of Thermotolerance.  We studied the pattern of expression of three $HSP-70$ genes in a fibrosarcoma cell line (HT1080) and a lung adenocarcinoma cell line (A549) following a primary heat dose of 45°C for 20 min (HT1080) or 45°C for 15 min (A549), reducing the surviving fraction to 0.5. Messenger RNA synthesis was then correlated with the development and decay of thermotolerance as cells were incubated at 37°C for up to 120 h (Fig. 1, a and b and Fig. 2, a and b). In all cell lines, there was a dramatic induction of transcription of all genes following heat shock. However, the total length of time for which the respective mRNAs continued to be expressed differed. As Figs. 1a and 2a indicate, $70A$ expression best correlates with the development and decay of thermotolerance. In HT1080 cells, $70A$ was maximally expressed between 2 and 24 h and was back to the control level by 72 h. This closely correlated with thermotolerance, which was maximum at 4–24 h and decayed to control levels by 72 h. $70B'$, on the other hand, was maximally expressed between 2 and 4 h and was back to control level by 48 h. For $70B$, the increased expression lasted as long as thermotolerance; however, the induction level was somewhat less compared to $70A$ and $70B'$. In A549 cells, the same patterns were seen with minor variations in the length of gene expression time (Fig. 2, a and b).

To investigate whether $HSP-70$ gene expression also correlates with the severity of the primary heat shock, HT1080 cells were treated at 45°C for 40 min (surviving fraction, $=0.1$). With this primary heat treatment, the thermotolerance of HT1080 cells was maximum at 24–48 h, began to decay by 48–72 h, and was almost at control level by 120 h (Fig. 3a). Fig. 3 also shows the expression of $HSP-70$ genes. The heat shock increased the

### Table 1 Sequences for primers and probes specific to $HSP-70A$, $HSP-28$, and $actin$

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer 1</th>
<th>Primer 2</th>
<th>Probe</th>
<th>Nucleotide position</th>
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<tr>
<td>HSP-70A</td>
<td>5'-CTAGGTGCTAGGGATCCGTGAGAC-3'</td>
<td>5'-GTTCCCTGTCTCTGCTGAGCTGTGTT-3'</td>
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<td>(406–429)</td>
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<td>5'-GCGGAGGTGTAATCTGGAATGAC-3'</td>
<td>5'-AGTGGAGGTCCACCTGCTGTTTCT-3'</td>
<td>(111–123)</td>
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<td>(134–160)</td>
</tr>
<tr>
<td>HSP-70B'</td>
<td>5'-CTCAACTACGTAGGGGCTGGCTGTTTTT-3'</td>
<td>5'-ATTTAGGTCTATATACATATTTTTAC-3'</td>
<td>5'-TAAAGTGCACAAAGTTTATTTATAAATT-3'</td>
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<td>(2245–2274)</td>
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<tr>
<td>HSP-28</td>
<td>5'-CCGGTGCACTGGCGCCTGCTGCTGCTGAC-3'</td>
<td>5'-CCGGGGAGATGTGAGAACCAGTTGGCTGCTGAC-3'</td>
<td>5'-GTTGATCTCCACACCGCATCCTGAC-3'</td>
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<td>Actin</td>
<td>5'-AGGCCAACCGGGAGAAGATGATGACC-3'</td>
<td>5'-CACAGTGAGCGCGGGGCACCTGAAC-3'</td>
<td>5'-TAGTACAAACTCTTGGAAGTT-3'</td>
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<td></td>
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<td>(2379–2401)</td>
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since this spot is enhanced following heat shock. The basic spot b, which is strictly heat induced, might be 70B'. Spot a, which is enhanced after heat shock, likely corresponds to the gene product of HSP-70B. An HSP-28 spot cannot be clearly identified. This is consistent with the very low induction of HSP-28 mRNA observed in HT1080 cells. The decay pattern of the HSP-70 spots correlates with the decay of the corresponding mRNA and thermotolerance. Note that by 48 h (spot b) and 72 h (spots a and c) the protein levels are back to control levels.

Kinetics of Expression of 70A, 70B, 70B', and HSP-28 after Heat Shock in Leukemic Cells. KG-1 and HL-60 cells were heat shocked at 42°C for 40 min (surviving fraction, 0.5). The cells were then incubated at 37°C for up to 48 h, total RNA was isolated, and PCR was performed (Figs. 5 and 6). Both KG-1 and HL-60 cells developed thermotolerance that was maximum by 8 h and decayed to control levels by 24 or 48 h. The 70B gene was constitutively expressed with little or no induction following heat shock. Expression of 70B' was induced for only 2 h. Both the 70A and HSP-28 genes showed a longer period of expression and a positive correlation with the development and decay of thermotolerance. However, for HSP-28, there was a delay in the expression for at least 2–4 h following heat shock (Figs. 5b and 6b).

length of time for each gene's expression; 70A expression again showed the best correlation with the development and decay of thermotolerance. 70A expression was maximum at 2–48 h, began to decay at 48 h, and was almost at control level by 120 h. 70B' expression was maximum at 2–24 h; however, the expression level was at control by 72 h.

The expression of the HSP-28 gene following 45°C, 20-min primary heat shock was also investigated in HT1080 cells. However, HSP-28 was only slightly induced at 24 h following such heat treatment in this cell line. HSP-28 gene expression is severely inhibited when the primary heat dose is at 45°C.4

To investigate whether we can distinguish the particular spots on a 2-dimensional gel as specific HSPs and whether the decay pattern correlates with the decay of thermotolerance, we performed the following studies. HT1080 cells were given a 45°C, 20-min heat shock and then labeled with [3H]leucine for 8 h. The radioactivity was then chased by incubating the cells at 37°C for up to 96 h. The protein profile was then investigated using 2-dimensional gel electrophoresis. As Fig. 4 indicates, there are several HSP-70 spots which are either enhanced or induced after heat shock. It is not very clear which spot corresponds to which HSP-70 gene. However, spot c may be 70A,
Kinetics of Gene Expression Following Treatment of Leukemic and Nonleukemic Tumor Cells with Sodium Arsenite. Arsenite treatment (100 µmol, 1 h at 37°C) of nonleukemic cells (HT1080) induced 70B and 70B' for 2-4 h. As was the case with heat, however, 70A expression correlated best with the development and decay of thermotolerance. Thermotolerance in HT1080 cells (Fig. 7) was maximum at 4-8 h following arsenite treatment and decayed to control by approximately 24 h. 70A expression was maximum between 2 and 8 h and was almost at control level by 24 h. On the other hand, while 70B' expression was maximum between 2 and 4 h, it returned to control levels by 8 h. Similarly, 70B expression was maximum 2 h posttreatment and decayed to almost control level by 8 h. In the leukemic cell line KG-1, there was a 2-h induction of 70B and 70B' post-arsenite treatment (100 µmol, 1 h at 37°C), but 70A expression again correlated best with the kinetics of tolerance (Fig. 8). The amount of thermotolerance in KG-1 cells post-arsenite treatment was only minor, with tolerance peaking at 4–24 h. 70A expression was also maximum between 2 and 8 h and reached control level by 24 h. Following arsenite treatment, HSP-28 was induced in both KG-1 and HT1080 cells. In KG-1 cells, HSP-28 expression was not evident for 2–4 h and then continued to be expressed even at 48 h, when thermotolerance was no longer evident. Not only was expression of HSP-28 in HT1080 cells delayed for 4 h following the termination of arsenite treatment, but there was only minor induction that continued for 72 h (data not shown). Correlation between 70A, 70B, and 70B' Expression and the Development and Decay of Thermotolerance. Figs. 9 and 10 show the results when the surviving fraction was plotted versus the level of expression (percentage of maximum in the x-axis) in each figure represents the total 32P count in each lane divided by the counts in the lane, which shows the maximum counts x100) of 70A, 70B, and 70B' for nonleukemic and leukemic tumor cells after heat or arsenite treatment. For the nonleukemic tumor cells, 70A, 70B, and 70B' show a positive correlation between the relative levels of mRNAs and survival level following various treatments. In Figs. 1, 2, 3, and 7 RNA levels appear to show a better correlation for 70A. As Fig. 9 indicates, there seem to be more changes in the level of mRNA expression for 70A (compared to 70B') during the development and decay of tolerance. 70B' mRNA expression is highest at very early times following heat shock and decays faster than 70A following similar heat treatment. Perhaps the expression of 70B' is responsible for the early events occurring during thermotolerance development. The correlation between the levels of mRNA and survival of 70B is similar to that of 70A, but with more variations in the mRNA levels at higher survival levels. In general, as the data points in Fig. 9 indicate, small changes in the level of HSP-70 mRNA result in dramatic changes in survival during
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Fig. 5. Surviving fraction and expression of HSP-70A, 70B, 70B', HSP-28, and actin following 42°C for 40 min in KG-1 cells. For survival studies, cells were heat shocked at 42°C for 40 min. They were then incubated at 37°C for increasing time intervals before being given a challenging dose of 45°C for 15 min and assayed for survival. For PCR studies, after heat shock, cells were incubated at 37°C for increasing time intervals, they were then harvested, total RNA was isolated and quantitated, and 1 µg of total RNA was then used in the amplification procedure. O/C (untreated control), 2, 4, 8, 24, and 48, hours at 37°C following heat shock. Ordinate, 32P count obtained from AMBIS of each hybridized filter. The lengths of the amplified product in base pairs are 204, 110, 207, 255, and 332 for 70A, 70B, 70B', HSP-28, and actin, respectively.

Fig. 6. Surviving fraction and expression of HSP-70A, 70B, 70B', HSP-28, and actin following 42°C for 40 min in HL-60 cells. Cells were heat shocked at 42°C for 40 min. For survival studies, they were then incubated at 37°C for increasing time intervals before being given a challenging dose of 45°C for 15 min and assayed for survival. For PCR studies, after heat shock, cells were incubated at 37°C for increasing time intervals, they were then harvested, total RNA was isolated and quantitated, and 1 µg of total RNA was then used in the amplification procedure. O/C (untreated control), 2, 4, 8, 24, and 48, hours at 37°C following heat shock. Ordinate, 32P count obtained from AMBIS of each hybridized filter. The lengths of the amplified product in base pairs are 204, 110, 207, 255, and 332 for 70A, 70B, 70B', HSP-28, and actin, respectively.

The molecular mechanisms responsible for the phenomenon of thermotolerance are not completely understood. What is known is that a variety of systems demonstrate a positive correlation between the concentration of HSP-70 and thermotolerance development after heat shock. Furthermore, agents known to enhance HSP synthesis induce thermotolerance, and agents known to induce thermotolerance enhance HSP synthesis. Additional evidence has been presented by Johnston and Kucey (22). These investigators competitively inhibited the heat-inducible expression of intact HSP-70A mRNA in Chinese hamster ovary cells by inserting the 5' control region of HSP-70 gene on a plasmid containing the dihydrofolate reductase gene. Following transfection of Chinese hamster ovary cells with such plasmids, cells showed increased sensitivity to acute heat shock. The microinjection of antibody against HSP-70 has also been shown to render rat cells more sensitive to acute heat shock (23).

We attempted to answer two questions. Is there a positive correlation between any of the heat-inducible HSPs and the development and decay of thermotolerance? Could the lack of induction and maintenance of thermotolerance in leukemic cells be explained by a lack of induction of one or more HSPs? Our results show that in nonleukemic cells (Fig. 9), the expression of all the HSP-70 genes correlates with the kinetics of thermotolerance; expression of HSP-70A correlates best. The lack of proper induction of the HSP-28 gene following heat shock in HT1080 cells suggests that HSP-28 may not be necessary for the induction of thermotolerance in these cells. It should be mentioned that the transfection of both HSP-70A and HSP-28 genes has recently been reported in mammalian cells and that in both cases the overexpression of these genes resulted in increased survival (6, 7). Thus the expression of either HSP-28 or HSP-70 is sufficient for the induction of tolerance (heat resistance).

In leukemic cells the HSP-70s were either insignificantly induced or, if induced, maintained only for short periods of time. This correlates with the limited thermotolerance seen in the induction and decay of thermotolerance. A certain level of HSP-70 is sufficient, however, to maintain thermotolerance at its maximum, and beyond a certain level of mRNA accumulation there is no longer a change in the survival level. The straight lines in Fig. 9 show the exponential fit for the data points presented.

Fig. 10 shows similar results for leukemic cells. It is not clear whether the expression of any of these genes correlates with changes in the survival levels following heat or arsenite treatment, although it appears from Figs. 5, 6, and 8 that 70A more positively correlates with the kinetics of thermotolerance in leukemic cells than does the expression of other genes. The small amount and short duration of expression of heat shock genes following heat or arsenite treatment in leukemic cells is consistent with the limited amount and short duration of thermotolerance.

DISCUSSION

The molecular mechanisms responsible for the phenomenon of thermotolerance are not completely understood. What is known is that a variety of systems demonstrate a positive correlation between the concentration of HSP-70 and thermotolerance development after heat shock. Furthermore, agents known to enhance HSP synthesis induce thermotolerance, and agents known to induce thermotolerance enhance HSP synthesis. Additional evidence has been presented by Johnston and Kucey (22). These investigators competitively inhibited the...
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Fig. 7. Surviving fraction and expression of HSP-70A, 70B, 70B', and actin following sodium arsenite treatment of HT1080 cells. Cells were treated with 100 μmol of sodium arsenite at 37°C. They were then washed once with PBS and incubated at 37°C for increasing time intervals. For survival studies, they were incubated at 37°C for increasing time intervals before being given a challenging dose of 45°C for 80 min and assayed for survival. For PCR studies, after heat shock, cells were incubated at 37°C for increasing time intervals, they were then harvested, total RNA was isolated and quantitated, and 1 μg of total RNA was then used in the amplification procedure. 0/C (control), 2, 4, 8, 24, 48, and 72, hours at 37°C following arsenite treatment. Ordinate, 3P count obtained from AMBIS of each hybridized filter. The lengths of the amplified product in basepairs are 204, 110, 207, and 332 for 70A, 70B, 70B', and actin, respectively.

Among the HSP-70 genes, the expression of 70A correlated best with the duration of thermotolerance. 70B was constitutively expressed in leukemic cells but was not induced after heat shock. The expression of HSP-28 following heat shock correlated with the induction of thermotolerance in KG-1 cells. Following arsenite treatment, however, HSP-28 continued to be expressed 72 h later when thermotolerance was no longer present. Sodium arsenite possibly induces differentiation of KG-1 cells that may result in the overexpression of HSP-28.

It should be kept in mind that in all these studies, the relative levels of mRNA are being compared and not the protein products of these various genes. It is indeed possible that the turnover rates of various HSPs differ and that such proteins are ultimately responsible for the induction and maintenance of thermotolerance. It is, however, generally valid to assume that as long as the mRNA is expressed for any gene, the protein product for that particular gene is also being expressed. It has been shown that 70A mRNA is stabilized following heat shock (24); the half-life of 70A mRNA changes from 2 h prior to heat shock to over 7 h following heat shock. Similar studies have not been performed for the other M, 70,000 heat shock genes. It is apparent from our studies that following heat shock, 70A mRNA (at least for nonleukemic tumor cell lines) has a longer half-life than either 70B or 70B'. Furthermore, 70A mRNA seems to disappear faster in leukemic cells than in nonleukemic tumor cells after heat shock. This may indicate that 70A mRNA may not be stabilized in leukemic cells following heat shock. In the case of the HSP-28 gene, it has been shown previously that the protein product of this gene is phosphorylated immediately following heat shock (7). It has also been suggested that phosphorylation of the preexisting protein for HSP-28 rather than...
newly synthesized protein may be necessary for thermotolerance development. We have found, however, that HSP-28 mRNA is not expressed constitutively in the non-heat-shocked cells.

In conclusion, these studies indicate that there is a positive correlation between the levels of mRNA coding for HSP-70s and the development and decay of thermotolerance. Furthermore, the limited development of thermotolerance in leukemic cells also is reflected in the short time that the mRNA of most HSPs is expressed following heat shock.

REFERENCES

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