Lower Heat Shock Factor Activation and Binding and Faster Rate of HSP-70A Messenger RNA Turnover in Heat Sensitive Human Leukemias

Nahid F. Mivechi, Honghai Ouyang, and George M. Hahn

Department of Radiation Oncology, Cancer Biology Research Laboratory, Stanford University School of Medicine, Stanford, California 94305

ABSTRACT

Normal bone marrow progenitors and some leukemic cells develop only a limited amount of thermotolerance. Further, once developed, thermotolerance decays at a faster rate than that normally observed in cells of nonhemopoietic origin. Thermotolerance induction and maintenance correlates with reduced levels of expression of various Mr 70,000 heat shock proteins (HSP-70) mRNAs after heat shock. We have now compared the accumulation of HSP-70 proteins in heat-shocked human leukemia cells KG-1, HL-60, and K562 to that in Ht1080, a colon carcinoma cell line. We have found reduced accumulation of HSP-70 proteins in all leukemic cells. The rate of decay of HSP-70A mRNA, measured following heat shock by using actinomycin D treatment to inhibit further RNA synthesis, was more rapid in KG-1 and HL-60 cells compared to Ht1080 cells. The half-life of HSP-70A mRNA was 2 h in KG-1 and HL-60 cells while in Ht1080 cells it was >7 h. HSP-70A mRNA is known to decay with a half-life of 2 h in unheated cells; this is increased to >7 h following heat shock. We therefore postulate that leukemic cells lack the mechanism to stabilize HSP-70A mRNA after heat shock. One postulated mechanism for HSP-70 mRNA decay rate is known to be due to the nucleotide sequences at the 3'-untranslated region. We examined the 3'-untranslated region in leukemic cells. No sequence variations, however, were observed at either the genomic or the complementary DNA levels between leukemic or nonleukemic tumor cells. Heat shock factor activation and binding by gel retardation assays showed that KG-1 and HL-60 cells had a reduced heat shock factor binding to the heat shock element when compared to K562 and Ht1080 cells. Furthermore, HSF-1 mRNA was found to be expressed at relatively lower levels in HL-60 cells when compared to Ht1080 or KG-1 cells. In conclusion, reduced HSP synthesis and accumulation of leukemic cells after heat shock correlates with the reduction in heat shock factor binding and a faster HSP-70A mRNA decay rate that is observed in these cells.

INTRODUCTION

Almost all mammalian cells develop transient heat resistance (thermotolerance) after a nonlethal heat shock (1, 2). The mechanism of thermotolerance is not yet fully understood. The Mr 70,000 family of HSPs3 has been postulated to play a role in its development and maintenance (3–5). During thermotolerance, HSP-70 may bind and prevent denaturation or help proper refolding of heat labile proteins (6).

Although thermotolerance development is observed in almost all cell types, normal bone marrow progenitors and some leukemic cells are anomalies; they do not develop significant amounts of thermotolerance (7–11). Leukemic cells exhibit a very short period of enhanced synthesis of mRNA for several HSP-70 genes after heat shock, they also accumulate over 100-fold lower amounts of HSP-70 mRNA after heat shock when compared to non-leukemic tumor cells.4 The reason as to why normal bone marrow progenitors and leukemic cells do not synthesize significant amounts of heat shock proteins is most likely due to the developmental regulation of HSP-70 gene expression (12, 13). Bone marrow stem cells and leukemic cells derived from them show reduced or absence of heat shock protein synthesis constitutively and in response to heat shock. This may explain the increased heat sensitivity and possibly also relate to the X-ray and drug sensitivity of normal bone marrow progenitors.

We now show that the lack of a full thermotolerance response on the part of KG-1 and HL-60 cells may be due to the reduced levels of HSP-70 mRNA accumulation after heat shock. These cells also show reduced activation of HSF binding to the HSE after heat shock. Further, mRNA coding for HSP-70A does not appear to be stabilized after heat shock, as it is in nonleukemic cells (14).

MATERIALS AND METHODS

Cell Culture and Maintenance

K562 (15) is a chronic myelogenous leukemic cell line, and HL-60 (16) and KG-1 (17) are acute myelogenous leukemic cells; Ht1080 is a colon carcinoma cell line. All cell lines were obtained from the American Type Culture Collection. Leukemic cells were maintained in Iscove’s minimal essential medium plus 20% FCS. Ht1080 cells were maintained in α-minimal essential medium plus 10% FCS. For HSF activation and binding studies, however, Ht1080 cells were also grown in Iscove’s medium plus 20% FCS and the data (data not shown) were similar to those shown in Fig. 4.

Actinomycin D Treatment

Ht1080, KG-1, and HL-60 cells were treated with 2 or 1 μg (in the case of KG-1 and HL-60) of actinomycin D/ml of medium for the appropriate times. [3H]Uridine incorporation studies indicated inhibition of mRNA synthesis by 90% or more after 1 or 6 h of actinomycin D treatment. The concentrations of actinomycin D that were used here caused minimal cytotoxicity.

Enzyme-linked Immunosorbent Assay

Following hyperthermia treatment, cells were washed with PBS; 0.5 ml of 1 mm Mg2+-ATP was added to each sample. Cells were disrupted by sonication the cell lysates were microfuged, and the supernatants were used in quantitation of proteins and ELISA assays. Protein was quantitated by using the BCA protein assay kit (Pierce, Rockford, IL). Different concentrations (0.25–2 μg) of proteins from each sample were then aliquoted in 96-well ELISA plates. After the overnight incubation at 4°C, the wells were washed with PBS. Appropriate dilutions of the antibody against HSP-70 (N27) were added to each well. After 2 h at 37°C, wells were washed with PBS plus 0.1% Tween 20. Wells were then treated with the appropriate dilution of horseradish peroxidase-linked anti-mouse antibody and incubated at 37°C for 2 h, the plates were washed and stained and were quantitated by an ELISA reader. The relative absorbance was calculated as per 1 μg of protein for each sample (18).

1 This work was supported by NIH Grants CA-54093 (N. F. M.) and PO1 CA 44665 (G. M. H.).
2 To whom requests for reprints should be addressed.
3 The abbreviations used are: HSF, heat shock factor; HSE, heat shock element; HSP, heat shock protein; HSP-70, Mr 70,000 heat shock protein; FCS, fetal calf serum; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; cDNA, complementary DNA.

References

1. 5/19/92; accepted 10/7/92.

2. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

3. The abbreviations used are: HSF, heat shock factor; HSE, heat shock element; HSP, heat shock protein; HSP-70, Mr 70,000 heat shock protein; FCS, fetal calf serum; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; cDNA, complementary DNA.


Downloaded from cancerres.aacrjournals.org on May 1, 2017. © 1992 American Association for Cancer Research.
One-Dimensional Gel Electrophoresis

Approximately 1–2 x 10^6 cells were labeled with [35S]methionine (40 μCi/ml; specific activity, > 6000 Ci/mmol; Amersham) in methionine-free minimal essential medium for 2 h, either before or after heat shock. At the end of the labeling period, cells were washed with PBS, lysed in sodium dodecyl sulfate sample buffer, and analyzed by PAGE as described previously (19). Equal cell numbers were loaded in each lane.

Competitive Quantitative PCR

Plasmids and Plasmid Construction. The plasmid pHSP-70 was obtained from Dr. R. Morimoto (Northwestern University). It contained the entire 2.3-kilobase fragment of the HSP-70A gene at Sphi-HindIII site (20). pHSP-70 was modified as follow: T7 promoter sense and antisense were synthesized by an automated DNA synthesizer and after hybridization, the double-stranded fragment was inserted at 5'-end of the HSP-70A gene at Sphi site.* This was done in order to enable the synthesis of mRNA in vitro by using the T7 polymerase. A double-stranded 23 base pair synthetic linker was also inserted at BamH1 (~340 from the 5'-end of HSP-70A gene) site between the primers 1 and 2 (described below). This was done in order to increase the size of the amplified fragment so that it can be distinguished from the amplified mRNA obtained from cells when this synthetic mRNA from the constructed plasmid pHSP-70L mRNA and mRNA from cells are mixed and amplified. The nucleotide sequence for the T7 promoter, linker, and the primers used to amplify HSP-70A mRNA have been previously described (7) and were as follow: T7 promoter (sense), 5'-TATAATAC-GACTCACATAGGG-3'; the T7 promoter antisense was complementary to the sense nucleotide sequence as above. The nucleotide sequence for the linker (sense) was 5'-GATCCCGGCTGAAGCTTCT-GACG-3'; primer 1 (sense) 5'-CTAGCCTGAGGAGCTGCTGCGA-
CATCACTTGA-3'. After 30 cycles of amplification, using either 1 μg of genomic or cDNA from various leukemic or nonleukemic cells, the products were precipitated with ethanol and analyzed by electrophoresis on an agarose gel (1.8%) containing ethidium bromide. Gels were scanned on an automated DNA sequencing, using the dideoxy chain termination method.

RESULTS

Reduced Synthesis and Accumulation of HSP-70 after Heat Shock in KG-1 and HL-60 Cells as Measured by PAGE Analysis and ELISA. KG-1 and HL-60, two leukemic cell lines, do not develop substantial amounts of thermostability when compared to nonleukemic tumor cell lines such as Ht1080 or A549 (7, 9). Further, compared to Hi1080 or A549 cells, HL-60 and KG-1 cells synthesize significantly less HSP-70A, HSP-70B, or HSP-70 mRNA after heat shock (7, 9). Studies using competitive quantitative PCR indicate that Ht1080 and A549 cells...
accumulate 50–100 ng of HSP-70A mRNA/μg of total RNA, while HL-60 and KG-1 cells accumulate 0.3–0.6 ng of HSP-70A mRNA/μg of total RNA 8 h following heat shock. We measured the rate of synthesis of HSP-70 by PAGE analysis (Fig. 1) and accumulation of HSP-70 by ELISA (Fig. 2) in Ht1080, K562, HL-60, or KG-1 cells after heat doses of 45°C, 20 min; 45°C, 10 min; 42°C, 40 min; or 42°C, 40 min, respectively (such heat doses reduced the survival level in all cell lines by 50% as measured by colony formation assays; Refs. 7 and 9). The reduced rate of synthesis of HSP-70 following heat shock (Fig. 1) resulted in the reduction in the accumulation of HSP-70 protein in such cells as measured by ELISA (Fig. 2) in both KG-1 and HL-60 cells. K562 and Ht1080 cells, however, continued HSP synthesis for longer times (PAGE analysis not shown), leading to intermediate or high levels of HSP-70 accumulation in K562 and Ht1080 cells, respectively. Such results are in agreement with previous data showing that K562 cells developed thermotolerance (9) more efficiently than either KG-1 or HL-60 cells (9).

HSP-70A mRNA Decays Faster in KG-1 and HL-60 Cells Than Ht1080 Cells following Heat Shock. To examine if the reduced HSP-70 accumulation in leukemic cells were due to a faster decay rate of HSP-70A mRNA, KG-1, HL-60, and Ht1080 cells received heat doses of 42°C, 40 min; 42°C, 40 min; or 45°C, 20 min, respectively. Cells were then incubated at 37°C for 1 h to allow the accumulation of HSP-70A mRNA and were then treated with 2 (Ht1080) or 1 (KG-1 and HL-60) μg of actinomycin D/ml of medium and then further incubated at 37°C for 1, 2, 4, 6, or 8 h before total RNA was isolated and HSP-70A mRNA was analyzed by competitive quantitative PCR. Fig. 3 shows the levels of HSP-70A mRNA after heat shock and subsequent actinomycin D treatment in Ht1080, KG-1, and HL-60 cells. As in other cells, the half-life of HSP-70A mRNA for Ht1080 cells was found to be more than 7 h (14), while that of KG-1 and HL-60 cells was about 2 h. It should be noted that the half-life of HSP-70A mRNA for HL-60 and KG-1 cells was measured within the first 4 h after actinomycin D treatment to avoid artifacts induced by the toxic effects of the drug. The half-life of HSP-70A mRNA has been shown to be 2 h before, and to stabilize to >7 h after heat shock (14). Our results suggest that KG-1 and HL-60 cells lack the ability to stabilize HSP-70A mRNA after heat shock. Fig. 3 (left) also shows the rate of actin mRNA decay for Ht1080, HL-60, and KG-1 cells. Actin mRNA was amplified by using actin specific primers described previously (7, 21). The amplified fragments were then detected by the 32P-labeled probe hybridized to the amplified product, using Southern blot analysis (7, 21). The blot was then counted on AMBIS. Actin mRNA was found to be rather stable within the first 6–8 h post-actinomycin D treatment. The autoradiographs shown in Fig. 3, right, are done by Southern blot analysis of the amplified product, using relative PCR with primers and probe specific to HSP-70A mRNA described before in detail (7, 21).

The rate of mRNA turnover in K562 cells were not measured. K562 cells are known to differentiate into erythroblasts in the presence of actinomycin D and, since differentiation often alters mRNA stability, we chose not to examine HSP-70A mRNA turnover in these cells.

3'-Untranslated Region of Leukemic and Control Cells. One possible mechanism of regulation of mRNA turnover resides at 3'-UT (25). We therefore, sequenced the 3'-UT regions spanning the codons 682 to 961 of either the genomic or the cDNA from HL-60, KG-1, K562, and HeLa cells or the plasmid pHSP-70. We found no sequence variations among any of these cell lines and those reported by Hunt and Morimoto (20), except the sequence contained an extra C at the 810-base pair.

Fig. 1. Rate of HSP synthesis after heat shock in KG-1 and HL-60 cells. KG-1 and HL-60 cells received a heat dose of 42°C, 40 min. Cells were then labeled with [35S]methionine for 2 h. After labeling samples were washed with PBS and solubilized in sodium dodecyl sulfate sample buffer and equal numbers of cells/lane were analyzed by sodium dodecyl sulfate-PAGE. C, control received no heat shock. Cells were incubated 0, 2, 4, 6, and 24 h at 37°C following heat shock before 2 h of labeling. Molecular weight markers are shown at the left.
position of HSP-70A gene. This was missing in all the cell lines and plasmids sequenced and most likely is an error in the original published sequence (20).

KG-1 and HL-60 Cells Show Reduced HSF Activation and Binding when Compared to Ht1080 Cells. The reduced HSP-70 mRNA synthesis (7) which was followed by the reduced accumulation of HSP-70 after heat shock in leukemic KG-1 and HL-60 cells indicated a possible lowered HSF activation and binding. Fig. 4 shows the HSF activation and binding to the HSE as analyzed by gel retardation assay in Ht1080 cells receiving a heat shock of 45°C, 20 min (surviving fraction, 0.5). Binding of activated HSF to the HSE was maximal immediately after heat shock and continued for at least 3 h. Although the data for Ht1080 has been shown as an example of a nonleukemic cell line, other nonleukemic tumor cells show basically similar HSF activation and binding (24, 26). A similar response was seen in K562 cells that received a 45°C, 10 min-heat shock (surviving fraction, 0.5) (Fig. 5). We observed reduced levels of HSF activation and binding to the HSE in KG-1 (Fig. 6) and HL-60 (Fig. 7) cells that received a heat dose of 42°C, 40 min (surviving fraction, 0.5). KG-1 cells show binding of activated HSF even in control cells (Fig. 6); however, the percentage of bound, activated HSF relative to the control does not increase after heat shock. Even after a more severe heat shock (45°C, 60 min) that reduced survival by 90% (Figs. 8 and 9), binding of HSF was still minimal in HL-60 cells, and increased only moderately in KG-1 cells. The variations in HSF activation among leukemic cells are most likely indicative of their stage of differentiation.

These results suggest that reduced binding of activated HSF is partly responsible for reduced accumulation of HSP-70 mRNA (and HSP-70 protein) in KG-1 and HL-60 cells.

We have further analyzed the expression of the human HSF-1 mRNA (Fig. 10) in both control and heat-shocked Ht1080, KG-1, and HL-60 cells. Our data indicate that HSF-1 mRNA is expressed less in HL-60 cells than in either Ht1080 or KG-1 cells. Further, the level of HSF-1 mRNA is constitutively expressed and is not induced after heat shock in any of the cell lines, as has been previously reported (22).

DISCUSSION

We have previously shown that bone marrow progenitors and some human leukemic cells do neither develop the same levels of thermostolerance nor levels of HSP-70 synthesis seen in nonleukemic tumor cells (8–10). We have now examined the possibility that the rapid degradation of HSP-70 mRNA or the inability of the HSF to bind to HSE is responsible for these findings.

The rate of HSP-70 mRNA decay is tightly regulated by a mechanism most likely determined by the 3'-untranslated region of the gene (25). This regulated turnover appears to determine HSP-70 mRNA accumulation at normal growth temperature (25). Following heat shock, HSP-70 mRNA is known to stabilize and its turnover rate decreases; the half-life of mRNA increases from 2 to >7 h (14). It has been postulated that the AT-rich regions at the 3'-UT region of HSP-70 gene most likely is the cause of such tight turnover regulation. This
is based on the finding that mRNAs, coding for c-myc, c-fos, and GM-CSF that also contain AT rich 3'-UT regions, all have very short half-lives (27). Furthermore, c-myc mRNA has been shown to be stabilized following heat shock (25). The precise mechanism of mRNA stabilization following heat shock is not entirely understood; besides the specific sequences at the 3'-UT regions, other mechanisms such as involvement of specific protein(s) or factor(s) which may stabilize or degrade mRNAs and are not functional or need to be resynthesized after heat shock cannot be ruled out (25).

Our results show that even after heat shock in KG-1 and HL-60 cells HSP-70A mRNA decays with a half-life of approximately 2 h. The rate of turnover of HSP-70A mRNA at the normal growth temperature in KG-1 and HL-60 cells is not
known because the levels are too low to be measured. If we assume that the half-life is similar to those in other cell types, then these mRNAs do not stabilize after heat shock. Possibly there is a lack of such regulated turnover in the leukemic cells. Our sequencing data showed no structural changes at the genomic or the cDNA levels at the 3'-untranslated region (3'-UT) of HSP-70A gene which could have explained faster decay rate of HSP-70A mRNA in leukemic cells. We sequenced the 3'-UT because HL-60 cells have been shown to carry abnormalities in several of their genes. These include amplified c-myc, mutated P53, and a large deletion in the GM-CSF gene (28). We did not find any abnormalities in the 3'-UT region. It is, of course, possible that KG-1 and HL-60 cells have gross abnormalities in other regions of the HSP-70A gene and these may contribute to the faster rate of HSP-70A mRNA decay in these cells following heat shock. The precise mechanism of mRNA stability is
not entirely understood; factor or factors contributing to mRNA stability other than sequence variations in the HSP-70 gene or cDNA may be altered in KG-1 and HL-60 cells.

HSF activation and binding studies showed low levels of HSF-HSE binding in heat-shocked KG-1 cells and almost an absence of activated HSF binding to HSE in heat-shocked HL-60 cells. The level of activated HSF-HSE in K562 cells was comparable to that of Ht1080 cells. However, although K562 cells normally show constitutive levels of expression for several of their HSP-70 genes (21), the total amount of HSP-70 protein accumulated after heat shock was lower in these cells than in Ht1080 cells. Such results may indicate a high rate of turnover
of HSP-70 mRNA in K562 cells as well. As was mentioned before, actinomycin D treatment of K562 cells induces these cells to differentiate (29) and differentiation itself might have an effect on mRNA turnover. Therefore, we chose to not study mRNA turnover in these cells. The low levels of activated HSF binding to HSE in HL-60 cells is interesting. The signal transduction pathway after heat shock that leads to HSF activation and binding is not understood. It is not inconceivable that HL-60 cells may have deficiencies in the pathway and, thus, be a vehicle for studying signal transduction. Although the data in Fig. 10 may indicate that HL-60 cells have reduced levels of HSF-1 mRNA, it is not known whether the activation of HSF is the key regulatory element in the heat shock response or that cells with lower levels of HSF-1 mRNA could also be deficient in exhibiting a heat shock response.

We studied the mechanism of heat shock response in leukemic cells because of their limited development of thermotolerance and its short durability. Our data suggest that mRNA turnover and/or reduced binding of HSF to HSE may be responsible. Whether these findings are modulated by the degree of differentiation of a particular cell line is not known at present. However, from the data presented here, it is likely that the developmental regulation of heat shock response that has been suggested previously in several systems may be at the level of expression and activation of heat shock transcription factor.

REFERENCES

Lower Heat Shock Factor Activation and Binding and Faster Rate of HSP-70A Messenger RNA Turnover in Heat Sensitive Human Leukemias

Nahid F. Mivechi, Honghai Ouyang and George M. Hahn