Identification of biological functions and gene networks regulated by heat stress in U937 human lymphoma cells

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Abstract. Although cancer cells exposed to temperatures >42.5˚C undergo cell death as the temperature rises, exposure of up to 42.5˚C induces slight or no cytotoxicity. The temperature of 42.5˚C is, therefore, well known to be the inflection point of hyperthermia. To better understand the molecular mechanisms underlying cellular responses to heat stress at temperatures higher and lower than the inflection point, we carried out global scale microarray and computational gene expression analyses. Human leukemia U937 cells were incubated at 42˚C or 44˚C for 15 min and cultured at 37˚C for 0-6 h. Apoptosis accompanied by the activation of caspase-3 and DNA fragmentation was only observed in cells treated with heat stress at 44˚C, but not at 42˚C. Although a large number of genes were differentially expressed by a factor of 2.0 or greater, we found substantial differences with respect to the biological functions and gene networks of the genes differentially expressed at the two temperatures examined. Interestingly, we identified temperature-specific gene networks that were considered to be mainly associated with cell death or cellular compromise and cellular function and maintenance at 44˚C or 42˚C, respectively, by using the Ingenuity pathway analysis tools. These findings provide the molecular basis for a further understanding of the mechanisms of the biological changes that are responsive to heat stress in human lymphoma cells.

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Abbreviations: AP-1, activating protein-1; ATF3, activating transcription factor 3; bZIP, basic-region leucine zipper; CCND1, cyclin D1; DNAJB1, DnaJ (Hsp40) homolog, subfamily B, member 1; DUSP1, dual specificity phosphatase 1; GADD45B, growth arrest and DNA-damage-inducible, β; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HMOX1, heme oxygenase (decycling) 1; HSF1, heat shock transcription factor-1; HSPA6, heat shock 70 kDa protein 6 (HSP70B'); HSPH1, heat shock 105/110 kDa protein 1; HSPs, heat shock proteins; IER3, immediate early response 3; JNK, protein kinases c-Jun N-terminal kinase; JUN, jun proto-oncogene; PML, promyelocytic leukemia; qPCR, quantitative polymerase chain reaction; RUNX1, runt-related transcription factor 1; SPP1, secreted phosphoprotein 1; SUMO1, SMT3 suppressor of mif two 3 homolog 1 (*S. cerevisiae*)

Key words: heat stress, microarray, gene function, gene network, apoptosis

Introduction

Hyperthermia is widely used to treat patients with various cancers, since tumor tissue is more sensitive to heat than normal tissue (1). Although cancer cells exposed to temperatures >42.5˚C alone will undergo cell death as the temperature rises, exposure of up to 42.5˚C called mild hyperthermia, alone induces slight or no cytotoxicity (2,3). The temperature at 42.5˚C is, therefore, known as the inflection point of hyperthermia, and generally up to 42.5˚C of mild hyperthermia is used in clinical cases in combination with other therapies including chemotherapy and radiotherapy (4-7).

In mammalian cells, heat stress induces a variety of stress responses such as an induction of heat shock proteins (HSPs), protein aggregation, an imbalance of protein homeostasis, DNA and RNA damage, reactive oxygen species production, cell growth arrest and cell death, including apoptosis (8-10). HSPs are highly conserved proteins whose expression is induced by different kinds of stresses, especially heat stress. HSPs behave as molecular chaperones for other cellular proteins and have strong cytoprotective effects preventing from cell death, including apoptosis and necrosis. HSPs achieve these effects by interacting with components of the cell signaling pathways, such as the caspase-dependent apoptosis, upstream and downstream of the mitochondrial level (11,12). It has been indicated that treatment of cells by heat induces numerous signal transduction pathways, which contribute to cell death and survival. Heat stress was reported to induce

apoptosis via activation of protein kinases c-Jun N-terminal kinase (JNK) (13,14). On the other hand, heat also activates extracellular signal-regulated kinases, and this activation provides cytoprotection from cell death by heating (15).

Recent DNA microarray and computational microarray analyzing technologies have provided a view of the expression profiles of tens of thousands of genes, and the relevant biological functions and gene networks based on the gene-expression data (16). Several investigators have indicated that heat stress affects the expression pattern of genes in many kinds of cell types with a cell death or no cell death condition (17-27). In addition, bioinformatics analysis have shown that differentially expressed genes influenced by heat could be sorted into several functional categories such as molecular chaperones, cell death, protein degradation, transcription, signal transduction and membrane transport (10,17,19,22,23,26). As described above, because heat stress induces complex cellular responses and changes in signal transduction, the mechanisms of heat response have not been elucidated in detail.

Our previous findings demonstrated that the development of apoptosis in human leukemia U937 cells treated with heat stress was dependent upon exposure time and temperature, and that the inflection point appeared to be between 42 and 43˚C (28). To better understand the molecular mechanisms underlying cellular responses to heat stress at higher and lower temperatures than the inflection point in U937 cells, we fully compared the differences in the gene expression patterns of cells under apoptotic conditions at 44˚C and non-apoptotic conditions at 42˚C for the same duration period (15 min) using global scale microarray and computational gene expression analysis.

Materials and methods

Cell culture and heat treatment. U937 cells, a human myelomonocytic lymphoma cell line, were obtained from the Human Science Research Resource Bank, Human Science Foundation (Tokyo, Japan). Cells were grown in RPMI-1640 medium (Sigma-Aldrich Co., Milwaukee, WI, USA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) at 37˚C. Heating was performed by immersing the 15 ml plastic tubes containing the suspended cells in a water bath at 42˚C (±0.05˚C) or 44˚C (±0.05˚C) for 15 min. The temperature was monitored with a digital thermometer (no. 7563, Yokogawa, Tokyo, Japan) during heating. After heat treatment, the cells were incubated for 0-6 h at 37˚C.

Analysis of apoptosis. Cells were lysed with a lysis solution containing 1 mM EDTA, 0.2% Triton X-100 and 10 mM Tris-HCl (pH 7.5) and centrifuged at 13,000 x g for 10 min. Subsequently, the DNA of the supernatant and of the pellet of each sample was precipitated with 12.5% trichloroacetic acid at 4˚C and quantified using a diphenylamine reagent after hydrolysis in 5% trichloroacetic acid at 90˚C for 20 min. The absorbance at 600 nm of each sample was determined after overnight color development with diphenylamine reagent. The percentage of fragmented DNA in each sample was calculated as the amount of DNA in the supernatant divided by the total-DNA of that sample (DNA in the supernatant plus pellet) (28).

Western blotting. Cells were dissolved in lysis buffer (50 mM NaCl, 1% Nonidet P-40 and 50 mM Tris-HCl, pH 8.0) containing a protease inhibitor cocktail (Nacalai Tesque, Inc., Kyoto, Japan). After electrophoresis on SDS-polyacrylamide gel, the proteins were transferred electrophoretically onto polyvinylidene fluoride membranes. The primary antibodies used were as follows: a rabbit polyclonal anti-Hsp70, a mouse monoclonal anti-Hsp40 and a rabbit polyclonal anti-Hsp27 antibody from MBL Co., Ltd. (Nagoya, Japan); a rabbit polyclonal anti-heat shock transcription factor-1 (HSF1) antibody (Stressgen Bioreagents Co., Ann Arbor, MI, USA); a rabbit polyclonal anti-caspase 3 antibody (Cell Signaling Technology, Inc., Beverly, MA, USA) and a mouse monoclonal anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (Organon Teknika Co., Durham, NC, USA). The anti-caspase 3 antibody recognizes the full length caspase-3 (35-kDa) as well as the fragments (19- and 17-kDa) of the activated enzyme. Immunoreactive proteins were visualized using a luminescent image analyzer (LAS-4000, Fujifilm Co., Tokyo, Japan) using an enhanced chemiluminescence detection system.

RNA isolation. Total-RNA was extracted from cells using an RNeasy total-RNA Extraction kit (Qiagen, Valencia, CA, USA), and treated with DNase I (RNase-Free DNase kit, Qiagen) for 15 min at room temperature to remove residual genomic DNA.

Microarray and computational gene expression analyses. Gene expression was analyzed using a GeneChip® system with a Human Genome U133-plus 2.0 array, which was spotted with approximately 54,000 probe sets (Affymetrix, Santa Clara, CA, USA). Samples for array hybridization were prepared as described in the Affymetrix GeneChip® Expression Technical Manual. The scanned arrays were analyzed using the GeneChip Analysis Suite Software (Affymetrix). The obtained hybridization intensity data were analyzed using the GeneSpring analysis software (Silicon Genetics, Redwood City, CA, USA) to extract the significant genes. To examine gene ontology, including the biological processes, cellular components, molecular functions, and gene networks, the obtained data were analyzed using the Ingenuity Pathways Analysis tools (Ingenuity Systems, Mountain View, CA, USA), a web-delivered application that enables the identification, visualization, and exploration of molecular interaction networks in gene expression data (29).

Real-time quantitative polymerase chain reaction (qPCR). Real-time qPCR assay was performed on the Mx3000P real-time PCR system (Stratagene Japan, Tokyo, Japan) using SYBR Premix Ex Taq (Takara Bio, Shiga, Japan) or Premix Ex Taq (for the use of TaqMan probes; Takara Bio) according to the manufacturer's protocol. Reverse transcriptase reaction (ExScript RT reagent kit, Takara Bio) was carried out with DNase-treated total-RNA using a random primer pd$(N)_6$. The primers and a probe (for HMOX1) were designed based on the following database sequences: AB066566, ATF3 (activating transcription factor 3); NM_006145, DnaJ (Hsp40) homolog, subfamily B, member 1 (DNAJB1); NM_004417, dual specificity phosphatase 1 (DUSP1); NM_015675, growth arrest and DNA-damage-inducible, β (GADD45B); NM_002046, GAPDH; NM_002133, heme oxygenase (decycling) 1 (HMOX1);

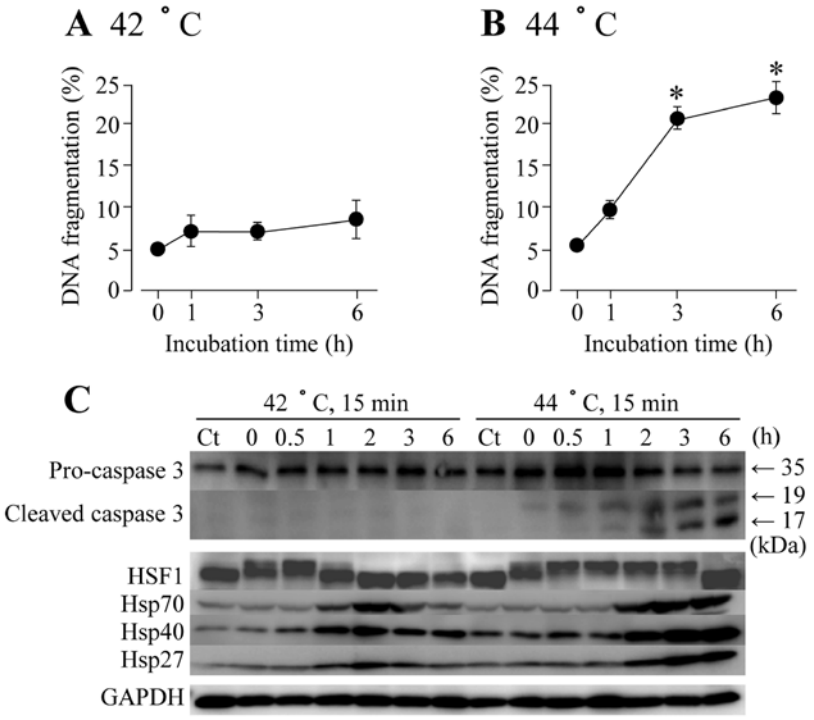

Figure 1. The effects of heat stress on apoptosis and the expression of HSPs and the HSF1 in human lymphoma U937 cells. Cells were exposed to heat stress at 42 or 44˚C for 15 min. After heat treatment, the cells were incubated for 0-6 h at 37˚C. DNA fragmentation (A and B) and caspase 3 activation (C) as indicators of apoptosis were measured. Protein expression level of HSPs and HSF1 was monitored (C). Data indicate the means ± SD of 6 different experiments. *P<0.05 vs. control; Ct, control.

NM_002155, heat shock 70 kDa protein 6 (HSP70B') (HSPA6); NM_006644, heat shock 105/110 kDa protein 1 (HSPH1). The specific primers and a probe for the jun proto-oncogene (JUN) were purchased from Applied Biosystems (Foster City, CA, USA). GAPDH was used as control for normalization (27).

Statistical analysis. Data are expressed as the means ± SD. Statistical analysis was carried out using the Student's t-test. P-values <0.05 were regarded as significant.

Results

The effect of heat stress on apoptosis and HSPs and HSF1 expression in U937 cells. To observe the effects of heat stress on the apoptosis of human lymphoma U937 cells, DNA fragmentation, a key feature of apoptosis, was examined. When U937 cells were treated with heat stress at 42˚C for 15 min followed by culture at 37˚C for 0-6 h, the percentage of DNA fragmentation did not change (Fig. 1A). However, heat stress at 44˚C for 15 min significantly increased DNA fragmentation, to 20.8±0.8% and 22.5±2.7% (mean ± SD) after 3 and 6 h of culture at 37˚C, respectively (Fig. 1B). This observation is in agreement with our previous study, in which the temperature threshold for the induction of apoptosis was 42-43˚C in U937 cells (28). Caspase-3 is a critical executioner of apoptosis, and proteolytic processing is required to generate the 19- and 17-kDa activated cleavages from its inactive 35-kDa full-length precursor during apoptosis (30). Western blot analysis in the present study demonstrated that the 19- and 17-kDa activated fragments of caspase-3 resulting from cleavage could be detected in cells treated with heat stress at 44˚C, but not at 42˚C (Fig. 1C). The data indicate that apoptosis was induced only in cells treated with heat stress at 44˚C.

In addition, the protein expression levels of HSF1 and HSPs were monitored by Western blotting (Fig. 1C). It is well known that the mobility shift of HSF1 due to its phosphorylation indicates the activation of the molecule (31). In U937 cells, the elevated expression of HSPs such as Hsp70, Hsp40 and Hsp27 was observed following the activation of HSF1 under both non-apoptotic and apoptotic conditions (19,26). At 42˚C for 15 min, a transient mobility shift of HSF1 was observed at 0 and 0.5 h after the treatment. A transient increase in the expression levels of Hsp70, Hsp40 and Hsp27 with a peak at 2 h after treatment was detected following HSF1 activation. In contrast, at 44˚C for 15 min, a sustained mobility shift of HSF1 was observed 0 to 3 h after the treatment. The expression levels of Hsp70, Hsp40 and Hsp27 gradually increased starting at 2 h after treatment. These data suggest a difference in the expression patterns of HSF1 and HSPs between the two experimental conditions (Fig. 1C).

Global gene expression analysis and identification of biological functions and gene networks. To identify the biological functions and gene networks regulated by heat stress, we carried out gene-expression profiling coupled to functional and signaling characterization by the Ingenuity software. Global-scale gene expression analysis of cells treated with heat stress identified many genes that were up- or down-regulated by a factor of 2.0 or greater. The total numbers of 810 and 2,124 probe sets were found to be differentially expressed as induced by heat stress at 42 or 44˚C for 15 min, respectively. We identified 163, 364 and 589 up-regulated and

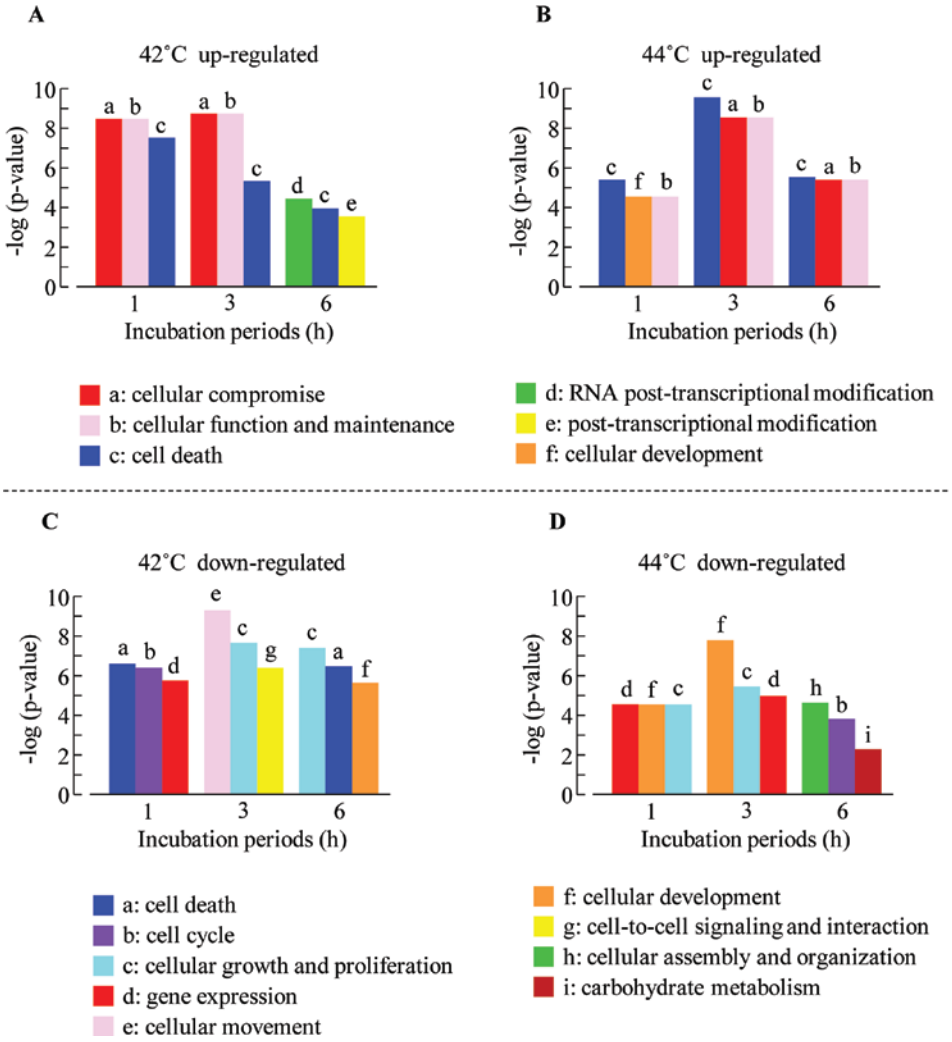

Figure 2. Functional category analysis. Functional category analysis of differentially expressed genes by a factor of 2.0 or greater in U937 cells treated with heat stress at 42 or 44˚C was performed using Ingenuity Pathways Analysis tools. The top 3 molecular functions were demonstrated in up- (A and B) and down-regulated genes (C and D).

95, 83 and 184 down-regulated probe sets at 1, 3 and 6 h after heat stress at 42˚C, respectively, and 280, 303 and 1069 up-regulated and 134, 469 and 674 down-regulated probe sets at 1, 3 and 6 h after heat stress at 44˚C, respectively. The complete list of genes from all samples has been stored in the Gene Expression Omnibus, a public database (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE23405).

In addition, functional category and gene network analyses were conducted by use of the Ingenuity Pathways Knowledge Base. We identified many functionally annotated genes; 69, 100 and 200 up-regulated and 45, 47 and 93 down-regulated genes at 1, 3 and 6 h after heat stress at 42˚C, respectively, and 81, 136 and 329 up-regulated and 67, 156 and 213 down-regulated genes at 1, 3 and 6 h after heat stress at 44˚C, respectively. The top three biological functions at each time point are summarized in Fig. 2. The biological functions including cellular compromise, cellular function and maintenance, and cell death were observed in the up-regulated genes at 42 or 44˚C, and the most remarkable functions were cellular compromise, cellular function and maintenance or cell death at 42 or 44˚C, respectively. In the down-regulated genes at both temperatures, the biological functions including gene expression, cellular growth and proliferation, and cellular development were observed. The specific biological functions were cell death and cellular movement or cellular assembly and organization and cell cycle at 42 or 44˚C, respectively. In addition, two significant gene networks, A and B, that were obtained from up-regulated genes at 42 and 44˚C 3 h post-treatment, respectively, were identified. The significant gene network A including many HSPs, cyclin D1 (CCND1) and runt-related transcription factor 1 (RUNX1) was mainly associated with cellular compromise and cellular function and maintenance (Fig. 3). At 42˚C, the peak gene expression of HSPs was observed at 3 h after heat stress, and the gene expression level of HSPs such as the Hsp70 (HSPA6 and HSPA1A), Hsp40 (DNAJA1 and DNAJB1) and Hsp27 (HSPB1) gene subfamilies was gradually elevated at 44˚C. These were consistent with the protein expression patterns of HSPs (Fig. 1C). In this network, the expression level of all genes was up-regulated at 42˚C, whereas those of 7 genes including CCND1, RUNX1 and promyelocytic leukemia (PML) were not altered at 44˚C (Fig. 3). The significant gene network B, including DUSP1, JUN, ATF3 and FOS, was mainly associated with cell death containing apoptosis. The

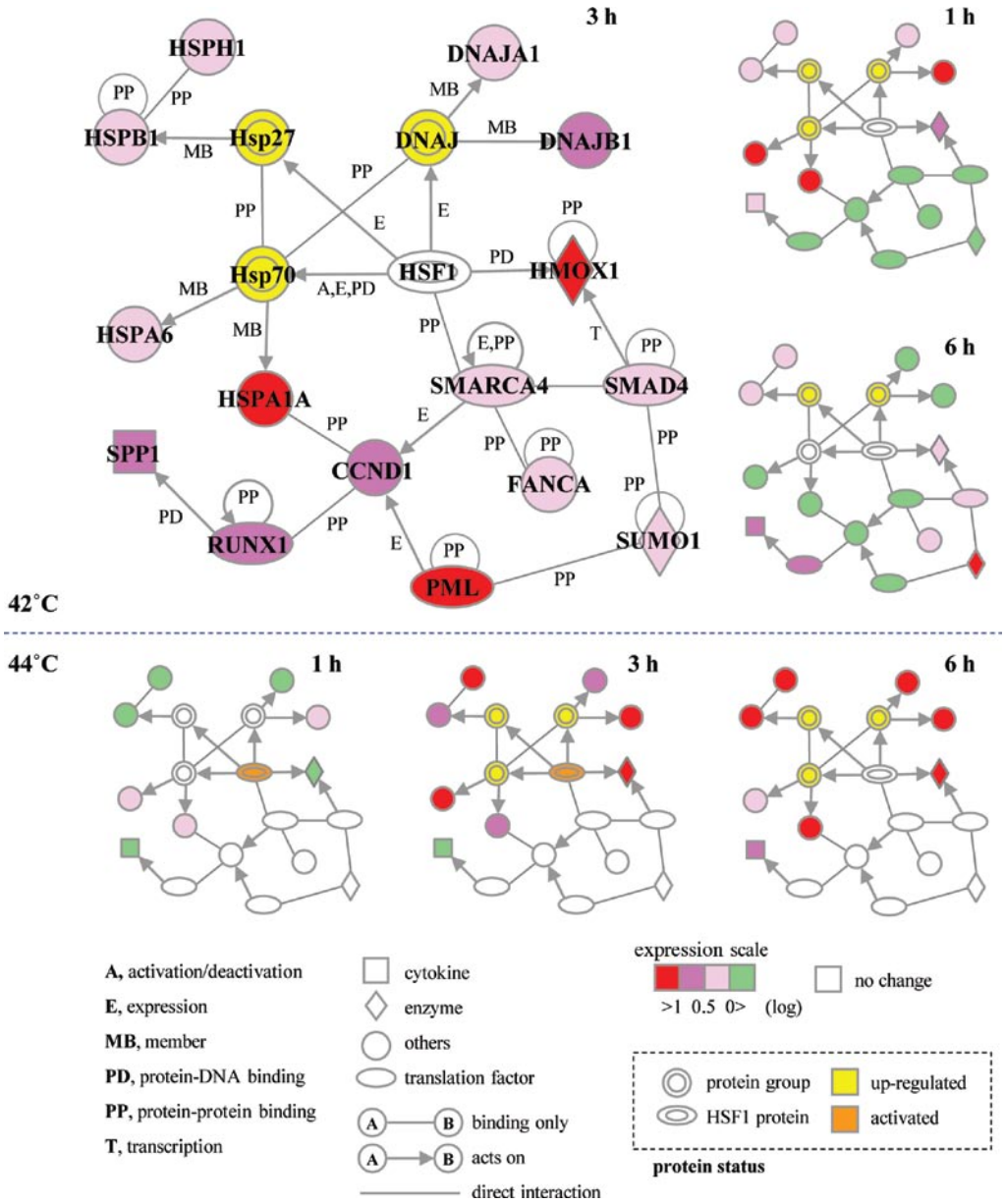

Figure 3. Gene network A. Up-regulated genes associated with cellular compromise and cellular function and maintenance 3 h after heat stress at 42˚C for 15 min were analyzed by Ingenuity Pathways Analysis tools. A time course of the gene expression of each network at 42 or 44˚C is shown. The network is displayed graphically as nodes (genes or proteins) and edges (the biological relationships between the nodes). The node color of the genes indicates the expression level of the genes. Nodes and edges are displayed by various shapes and labels that represent the functional class of genes and the nature of the relationship between the nodes, respectively. Protein status is shown on the basis of Western blot analysis data (Fig. 1C).

expression level of all genes in network B was increased 3 h after heat stress at 44˚C. On the other hand, only 5 of 19 genes in network B were found to be up-regulated at 42˚C (Fig. 4).

Quantitative analysis of differentially expressed genes. To further verify the alterations observed by microarray analysis, time-dependent changes at the mRNA level of selected genes that belonged to the gene networks A and B were monitored by using a real-time qPCR assay. Among the genes in gene network A, the expression levels of all genes such as DNAJB1, HMOX1, HSPA6 and HSPH1 were significantly increased, and the levels of DNAJB1, HSPA6 and HSPH1 for 1 and/or 3 h incubation at 42˚C were markedly higher than those at 44˚C (Fig. 5A-D). The expression levels of ATF3, DUSP1, GADD45B and JUN in gene network B for 1, 3 and/or 6 h incubation at 44˚C were markedly higher than those at 42˚C (Fig. 5E-H). These results are in very close agreement with those of the microarray experiments.

Discussion

In general, slight differences in the temperature and duration of heat treatment can produce variations in the cell death outcome. Although cancer cells with hyperthermia >42.5˚C will undergo cell death, slight or no cytotoxicity is induced by mild hyperthermia at <42.5˚C; 42.5˚C is therefore well known as the inflection point of hyperthermia (2,3). In our previous study, heat stress at 44-46˚C for more than 10 min of exposure

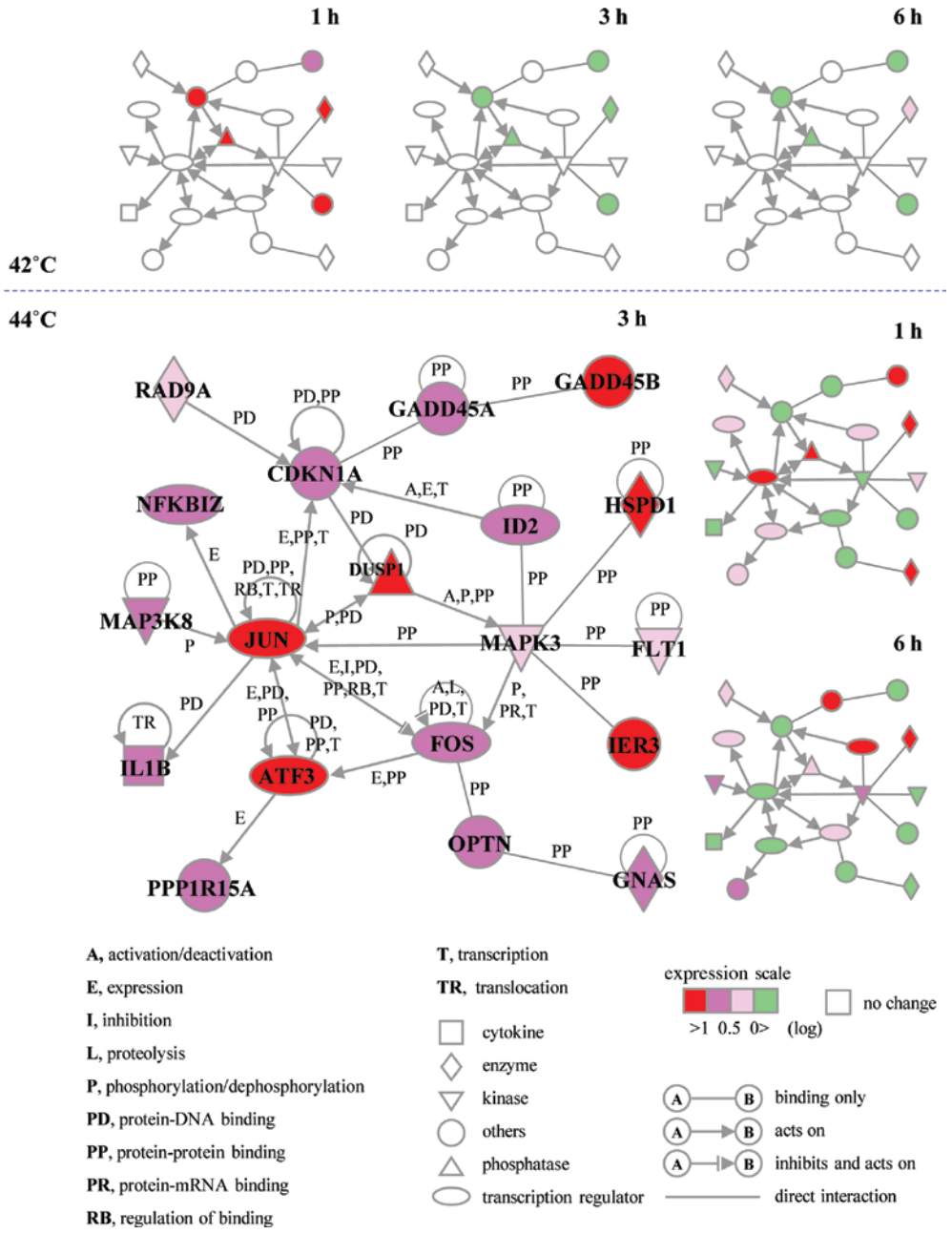

Figure 4. Gene network B. Up-regulated genes associated with cell death at 3 h after heat stress at 44˚C for 15 min analyzed by Ingenuity Pathways Analysis tools. A time course of the gene expression of each network at 42 or 44˚C is shown. For the explanation of the symbols and letters, see Fig. 3 legend.

markedly induced apoptosis, whereas heat stress at 41-42˚C for <30 min induced no cell damage in human leukemia U937 cells (20,26-28). On the other hand, treatment of U937 cells for relatively long periods of 60-120 min at 42˚C significantly elicited apoptosis (19,28). We have shown that the inflection point of apoptosis induced by heat stress appears to be between 42 and 43˚C in U937 cells (28). In the present study, heat stress at 44˚C but not 42˚C for 15 min significantly induced apoptosis accompanied by the activation of caspase-3 and DNA fragmentation in U937 cells. Furthermore, global scale microarray and computational gene expression analyses clearly demonstrated substantial differences in the biological functions of the differentially expressed genes and in the gene networks activated between the cells treated above and below the inflection point of heat stress.

Up to now, several investigators have found that the most powerful approach for elucidating details of the molecular mechanisms that are influenced by heat stress is to use microarrays and bioinformatics tools. Numerous genes and biological functions affected by heat have been thus identified (17-27). However, the discrepancy of these results seems to be due to different experimental conditions, including heating temperature, duration of heat treatment, the type of microarray, and origin of the cell. In this study, through the use of microarray analysis, we have added to the list of genes that are up- or down-regulated by more than 2.0-fold in U937 cells treated with heat stress under the non-apoptotic temperature of 42˚C and the apoptosis-inducing conditions at 44˚C. Moreover, functional analysis of the altered genes indicated that the molecular and cellular functional categories at higher and

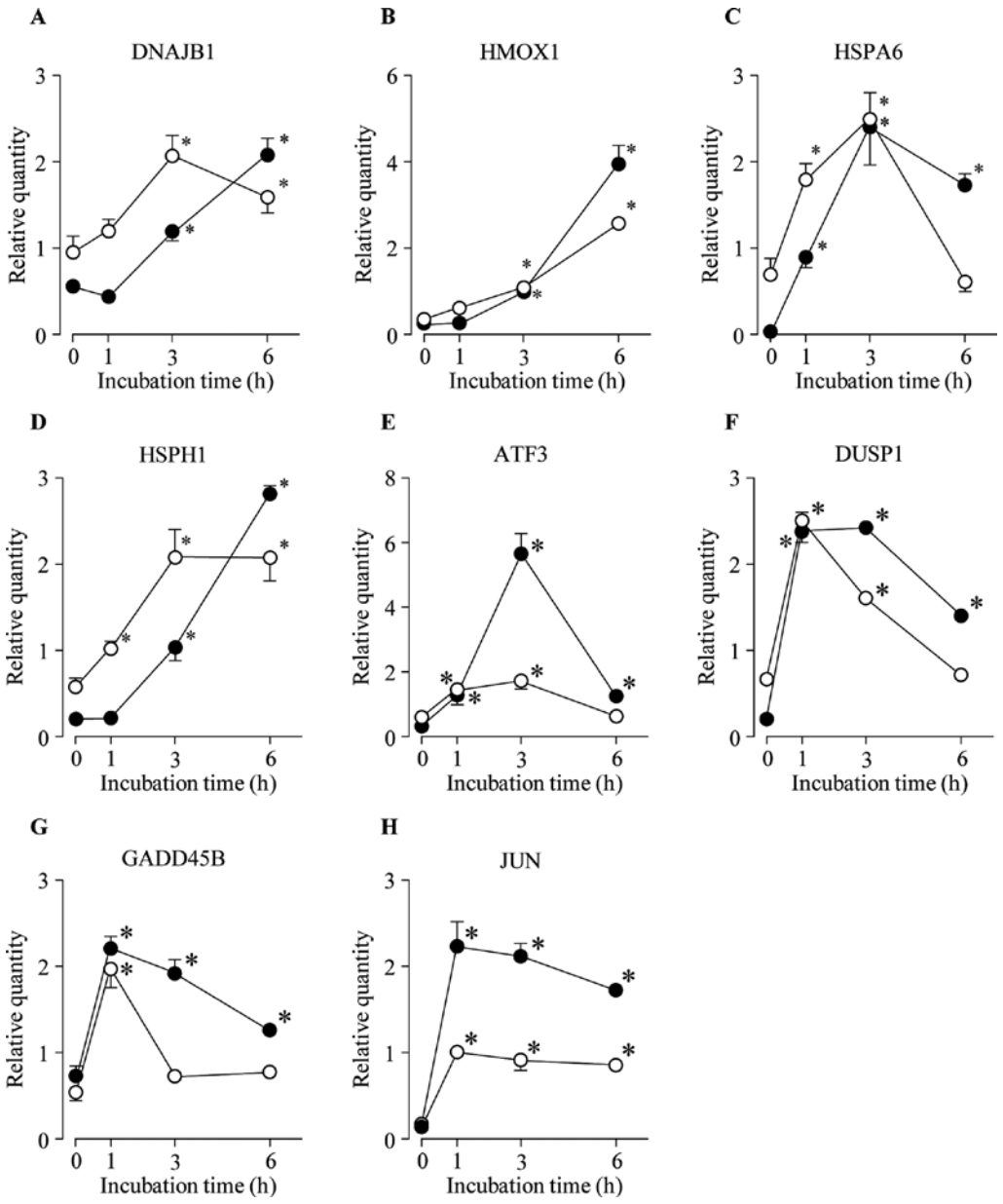

Figure 5. Verification of microarray results by real-time qPCR. U937 cells were incubated at 42 or 44˚C for 15 min and then cultured at 37˚C for 0-6 h. Real-time qPCR was carried out. DNAJB1 (A), HMOX1 (B), HSPA6 (C) and HSPH1 (D) or ATF3 (E), DUSP1 (F), GADD45B (G) and JUN (H) were included in gene network A or B, respectively. Each mRNA expression level was normalized to GAPDH expression level. Open circles, 42˚C; closed circles, 44˚C. Data are expressed as the mean ± SD (n=4). *P<0.05 vs. control (0 h).

lower temperatures than the inflection point of hyperthermia were strikingly different and exactly reflected each cell feature. Specifically, under a non-apoptosis-inducing protocol, the top biological functions were cellular compromise, while under apoptosis-inducing conditions they were cellular function and maintenance or cell death.

In order to gain further insight into the molecular mechanisms of both heat treatments, gene network analysis was performed. We successfully identified gene networks A and B obtained at the temperatures of 42 and 44˚C, respectively. In network A, containing the up-regulated genes, many HSPs, such as Hsp70 (HSPA), Hsp40 (DNAJ) and Hsp27 (HSPB1) were observed. Time-course changes of the expression of HSPs by microarray at both 42 and 44˚C were comparable to those from Western blot analysis and quantitative real-time PCR analyses. Induction of HSPs is a common response to heat with or without cell death, including apoptosis, as described in several microarray studies (17-19,21-27). HSPs have been shown to block apoptosis by interfering with caspase activation and to inhibit apoptosis directly or indirectly (11,12). HSPs under the present experimental conditions appeared to behave as anti-apoptosis molecules. However, as shown in Fig. 3 (gene network A) in addition to Fig. 1C and Fig. 5A, C and D, the induction of HSPs at 42˚C occurred at earlier time points than those at 44˚C, suggesting that the early heat shock response may be very important for preventing the progression of apoptosis. Gene network A also included HMOX1 and the secreted phosphoprotein 1 (SPP1), in agreement with previous reports (19,25,26). HMOX1 (32) and SPP1 (33) have been previously described as anti-apoptotic

molecules. In the present study, we observed 7 genes that were up-regulated only at 42˚C; of these, CCND1 (34), SMT3 suppressor of mif two 3 homolog 1 (*S. cerevisiae*) (SUMO1) (35), and RUNX1 (36) have been reported to inhibit apoptosis in fibroblast cells. Up-regulation of the expression of these 7 genes in addition to HSPs, HMOX1 and SPP1 may play an important role in the cytoprotection of U937 cells.

In this study, it is of particular interest that the significant and temperature-specific gene network B derived from genes that were up-regulated at 44˚C was mainly associated with the molecular function of cell death. In gene network B, although a transient increase in the expression of several genes including DUSP1, GADD45B and immediate early response 3 (IER3), was detected only after 1 h culture at 42˚C, the up-regulation of almost all genes responsive to heat stress at 44˚C was remarkable and relatively sustained. The expression profile of selected genes such as ATF3, DUSP1, GADD45B and JUN determined by microarray analysis exhibited a similar pattern of culture period dependence as determined by using real-time qPCR. DUSP1 (37) and GADD45B (38), previously reported to prevent apoptosis, were up-regulated under both non-apoptotic and apoptotic conditions and were determined to be in network B. Moreover, DUSP1 has been previously found to play an important role in inhibiting UV-induced apoptosis in U937 cells (37). Thus, these genes may be considered to participate in the cytoprotective action in U937 cells under the present experimental conditions. In network B, the expression of many genes, including the basic-region leucine zipper (bZIP) transcription factors JUN, ATF3 and FOS, were enhanced under only the apoptotic conditions at 44˚C. Homo- and heterodimeric protein complexes of bZIP proteins act as activators and repressors of transcription, and most commonly the JUN and FOS proteins form the heterodimer of the activating protein-1 (AP-1) complex (39). It has been reported that overexpression of JUN in combination with FOS increases the sensitivity of keratinocytes to apoptosis (40). ATF3 (41) and FOS (42) has been indicated to be associated with the induction of apoptosis. Ruiter *et al* (43) reported that a dominant-negative mutant of JUN inhibited radiation-induced apoptosis in U937 cells. It has been well known that the JNK pathway is involved in apoptosis evoked by heat stress (13,14). It is presumed that many temperature-specific genes, including the bZIP transcription factors expressed here, may participate in apoptosis by heat stress at 44˚C. In several microarray experiments, heat stress induced elevation of the expression of ATF3, DUSP1, FOS, GADD45B and JUN (17,19-22,25-27). Taken together, these results show that the temperature-specific and non-specific genes identified here that were differentially expressed and/or belonged to the gene networks, may participate in heat-stress responses with or without apoptosis in U937 cells.

In conclusion, these findings will provide the molecular basis for a further understanding of the mechanisms of the biological changes that are responsive to heat stress in human lymphoma cells. Hyperthermia and mild hyperthermia in combination with other treatments have been considered a promising approach in cancer therapy. Thus, the current data in addition to previous data will help enable the rational design of more effective strategies for future mild hyperthermia and hyperthermia therapy.

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