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Gene networks involved in apoptosis induced by hyperthermia in human lymphoma U937 cells

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Abstract

To define the molecular mechanisms that mediate hyperthermia-induced apoptosis, we performed microarray and computational gene expression analyses. U937 cells, a human myelomonocytic lymphoma cell line, were treated with hyperthermia at 42 °C for 90 min and cultured at 37 °C. Apoptotic cells (~15%) were seen 6 h after hyperthermic treatment, and elevated expression of heat shock proteins (HSPs) including Hsp27, Hsp40, and Hsp70 was detected, following the activation of heat shock factor-1. Of the 54,675 probe sets analyzed, 1334 were upregulated and 4214 were downregulated by >2.0-fold in the cells treated with hyperthermia. A non-hierarchical gene clustering algorithm, K-means clustering, demonstrated 10 gene clusters. The gene network U1 or U2 that was obtained from up-regulated genes in cluster I or IX contained HSPA1B, DNAJB1, HSPH1, and TXN or PML, LYN, and DUSP1, and were mainly associated with cellular compromise, and cellular function and maintenance or death, and cancer, respectively. In the decreased gene cluster II, the gene network D1 including CCNE1 and CEBPE was associated with the cell cycle and cellular growth and proliferation. These findings will provide a basis for understanding the detailed molecular mechanisms of apoptosis induced by hyperthermia at 42 °C in cells.

Keywords: Hyperthermia; Apoptosis; Gene expression; Gene network; Heat shock protein

1. Introduction

In medicine, hyperthermia has been a promising approach in cancer therapy. The temperatures used in heat treatments for 30–60 min are in the range of 40–45 °C in the case of locoregional treatment, and up to 42 °C in the case of whole-body hyperthermia (Hall, 2000). Regarding the molecular aspect of hyperthermia, heat shock proteins (HSPs) are highly conserved proteins in which synthesis can be induced by a variety of stresses, especially hyperthermia exposure. HSPs behave as molecular chaperones, which act as the primary cellular defence against damage to the proteome, initiating refolding of denatured proteins and regulating degradation after severe protein damage (Georgopoulos and Welch, 1993; Lindquist and Craig, 1988). HSPs protect cells both by limiting the effects of protein-damaging agents through protein chaperoning and refolding and by directly blocking the cell-death pathways (Beere, 2004). In addition, HSPs are crucial molecules in immune reponses and HSP chaperone tumor antigens are utilized for cancer immunotherapy based on hyperthermia (Ito et al., 2006; Ménoret and Chandawarkar, 1998; Srivastava et al., 1998). Although many biological processes are affected by hyperthermia, the overall responses to hyperthermia remain unclear.

DNA microarray technique has been used to identify changes in gene expression induced by mild hyperthermia at

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41 °C (Tabuchi et al., 2008) and hyperthermia at 43–44 °C (Borkamo et al., 2008; Hirano et al., 2005; Kato et al., 2003; Narita et al., 2002; Sonna et al., 2002; Wada et al., 2007; Zhou et al., 2007). Although this method is valuable in differential gene expression, there is an increasing need to move beyond this level of analysis. Recently, bioinformatics tools used to explore gene ontology and networks have been utilized to analyze functionally microarray data. Borkamo et al. (2008) have reported that gene ontology mapping can be used to sort 1213 differentially expressed genes into 10 main functional gene groups, including those related to apoptosis, transcription, and the immune system in rat glioma cells treated with hyperthermia at 43 °C. Our previous observations indicate that hyperthermia treatment at 42 °C of human leukemia U937 cells induced significant apoptosis, but this response followed mild hyperthermia treatment at 41 °C (Kameda et al., 2001). More recently, DNA microarray and gene network analyses demonstrated that significant gene networks were associated with cellular function and maintenance or cell morphology and the cell cycle in U937 cells treated with mild hyperthermia at 41 °C without inducing apoptosis (Tabuchi et al., 2008). To clarify the detailed mechanisms by which hyperthermia at 42 °C induces apoptosis in human leukemia U937 cells, we carried out a global-scale time-course microarray analysis using a GeneChip® analyzing system, and. explored the functional relationships among the candidate genes using Ingenuity Pathway Analysis tools.

2. Materials and methods

2.1. Cell culture

U937 cells, a human myelomonocytic lymphoma cell line, were obtained from the Human Science Research Resource Bank, Human Science Foundation (Tokyo, Japan). They were maintained as suspension cultures in RPMI1640 medium (Wako Pure Chemical Industries, Ltd., Osaka, Japan) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) at 37 °C in humidified air with 5% CO_2 and 95% air.

2.2. Hyperthermia treatment

Cells (3×10^6) were collected by centrifugation and suspended in 3 ml of fresh medium in plastic culture tubes before being exposed to 42 °C (±0.05 °C) for 0–120 min in a water bath. The temperature was monitored with a digital thermometer (No. 7563, Yokogawa, Tokyo, Japan) coupled to a thermocouple 0.8 mm in diameter during heating. After hyperthermia treatment, the cells were incubated for 0–6 h at 37 °C.

2.3. Analysis of apoptosis

For detection of DNA fragmentation, cells (3×10^6) were lysed with 0.2 ml of lysis buffer (1 mM EDTA, 0.2% Triton X-100 and 10 mM Tris–HCl, pH 7.5) and centrifuged at 13,000 × *g* for 10 min. Subsequently, each DNA in the supernatant and the pellet were precipitated in 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 in 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 (containing fragmented DNA) divided by total DNA for that sample (supernatant plus pellet) (Kameda et al., 2001).

For detection of the expression of phosphatidylserine outside the plasma membrane of cells, flow cytometry was used with propidium iodide (PI) and fluorescein isothiocyanate (FITC)-labeled annexin V (annexin V-FITC kit, Immunotech, Marseille, France). Briefly, cells (5×10^5) were suspended in the binding buffer of the kit. FITC-labeled annexin V and PI were added to the cell suspension. After incubation for 10 min at room temperature, cell suspensions were analyzed using a flow cytometer (Kameda et al., 2001).

2.4. SDS-polyacrylamide gel electrophoresis (PAGE) and western blotting

Cells were dissolved in a 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). SDS-PAGE and Western blotting were carried out as described elsewhere (Laemmli, 1970; Towbin et al., 1979). The primary antibodies used were as follows: a rabbit polyclonal anti-Hsp70 antibody (MBL Co., Ltd., Nagoya, Japan); a mouse monoclonal anti-Hsp40 antibody (MBL Co., Ltd.); a goat polyclonal anti-Hsp60 antibody (Santa Cruz Biotechnology, Inc., CA, USA); a rat monoclonal anti-Hsp90 antibody (MBL Co., Ltd.); a rabbit polyclonal anti-Hsp27 antibody (MBL Co., Ltd.); a rabbit polyclonal anti-Hsp110 antibody (MBL Co., Ltd.); a rabbit polyclonal anti-heat shock transcription factor-1 (HSF1) antibody (Stressgen Bioreagents Co., Ann Arbor, MI, USA); and a mouse monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (Organon Teknika Co., Durham, NC, USA). Immunoreactive proteins were visualized using a luminescent image analyzer (LAS-4000, Fujifilm Co., Tokyo, Japan) using an enhanced chemiluminescence detection system (Tabuchi et al., 2006a, 2008). The level of GAPDH was constant over the culture periods (supplementary data Fig. 1S A), and all the other protein expression levels were normalized to it.

2.5. RNA isolation

Total RNA was extracted from cells using an RNeasy Total RNA Extraction kit (Qiagen, Valencia, CA, USA) according to the manufacture protocol, and treated with DNase I for 15 min at room temperature to remove residual genomic DNA.

2.6. High-density oligonucleotide 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