Screening of stress enhancer based on analysis of gene expression profiles: Enhancement of hyperthermia-induced tumor necrosis by an MMP-3 inhibitor

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To improve the therapeutic benefit of hyperthermia, we examined changes of global gene expression after heat shock using DNA microarrays consisting of 12 814 clones. HeLa cells were treated for 1 h at 44°C and RNA was extracted from the cells 0, 3, 6, and 12 h after heat shock. The 664 genes that were up or down-regulated after heat shock were classified into 7 clusters using fuzzy adaptive resonance theory (fuzzy ART). There were 41 genes in two clusters that were induced in the early phase after heat shock. In addition to shock response genes, such as *hsp70* and *hsp40*, the stress response genes *c-jun*, *c-fos* and *egr-1* were expressed in the early phase after heat shock. We also found that expression of matrix metalloproteinase 3 (MMP-3) was enhanced during the early response. We therefore investigated the role of MMP-3 in the heat shock response by examining HeLa cell survival after heat treatment in the presence and absence of an MMP-3 inhibitor, N-isobutyl-N-(4-methoxyphenylsulfonyl)glycylhydroxamic acid (NNGH) or N-hydroxy-2(*R*)-[[4-methoxysulfonyl](3-picolyl)amino]-3-methylbutaneamide hydrochloride (MMI270). The number of surviving cells 3 days after heat treatment significantly decreased, reaching 3.5% for NNGH and 0.2% for MMI270. These results indicate that the MMP-3 inhibitors enhanced heat shock-induced cell death and behaved as stress enhancers in cancer cells. This valuable conclusion was reached as a direct result of the gene expression profiling that was performed in these studies. (Cancer Sci 2003; 94: 644–649)

Hyperthermia is now considered to be an effective modality for cancer therapy, particularly in conjunction with radiotherapy or chemotherapy. It is applied for two purposes: the direct induction of tumor cell death and the sensitization of cells to chemotherapeutics or anticancer agents. It is effective for intractable cancers, such as inoperable advanced cancer, radioresistant cancer and postoperative recurrent carcinoma. Heating methods that have been used in the clinic for this purpose include capacitive heating of tumors using a radiofrequency electric field and microwave hyperthermia.[1, 2)]

Major difficulties with the use of hyperthermia are the development of thermotolerance and the transient resistance induced by heat treatment or by prior sublethal heat treatment.[3)] Thermotolerance occurs when the cellular metabolism is maintained at elevated temperature and cell damage is repaired immediately. The induction of heat shock proteins (HSPs) by hyperthermia contributes to the acquisition of thermotolerance in tumor cells. Various studies[4–7)] have revealed a close relationship between the level of thermotolerance and the cellular content of HSP72, an inducible HSP70 family protein. For example, microinjection of antibodies against HSP70 makes cells more sensitive to thermal stress.[4)] In addition, HSP72 is associated with ribosomal subunits in thermotolerant cells, but not in normal cells.[5)] Furthermore, overexpression of HSP27 or alpha B-crystallin induces thermotolerance.[6, 7)]

We have developed hyperthermia using magnetite cationic liposomes (MCLs) which are submicron-diameter magnetic particles.[8, 9)] MCLs generate heat under an alternating magnetic field by hysteresis loss and thus mediate intracellular hyperthermia in cancer tissue. This type of hyperthermia can achieve uniform and exclusive heating of the tumor region without damaging the surrounding normal tissues. In an *in vivo* study using T9 rat glioma cells,[8)] an intracellular temperature that was high enough to induce tumor cell death was generated. Besides the heat stress, it was also found that an immune response was induced that caused complete regression of transplanted tumor tissue.[10)]

Recent reports have suggested the importance of HSPs, such as HSP70, HSP90 and glucose-regulated protein 96 (gp96), in immune reactions.[11, 12)] HSP-mediated anti-tumor immunity has been reported to cause a vaccine effect of HSP-peptide complexes released from human melanoma cells.[13)] Our experimental hyperthermia also induced a number of HSP70 proteins. In addition, we show that heat-induced expression of MHC class I antigen on the cells may be a mechanism for anti-tumor immunity induced by hyperthermia.[14)]

We have previously reported that leukocytes/lymphocytes such as CD3, CD4, CD8 and NK cells can be detected in the tumor tissues of rats that had been exposed to hyperthermia.[10)] Even if viable cancer cells remain in the tumor after hyperthermia, they will become targets for the anti-tumor immune reaction due to heat-induced expression of MHC class I antigen. However, there is a substantial number of tumor patients with low immunoactivity due to aging or emaciation. For such patients, hyperthermia is insufficiently effective even if sufficient heating is achieved. In such patients, the combination of heat stress enhancer with hyperthemia could be effective.

Recently, the HSP inhibitor I, N-formyl-3,4-methylenedioxybenzylidine-γ-butyrolactam, was shown to reduce the acquisition of thermotolerance,[15, 16)] and inhibitors of protein kinase C (PKC), such as staurosporine and 1-(5-isoquinolinesulfonyl)-2-methylpiperadine, have also been shown to suppress the development of thermotolerance.[17)] These results suggest that the combination of these chemicals with hyperthermia may be effective even in cancer patients with low immunoactivity. A better understanding of the mechanisms of the heat shock response should allow us to improve the effectiveness of hyperthermia, by interfering with the development of thermotolerance.

Recent technological developments, particularly in the area of gene expression profiling using macroarrays and microarrays, have greatly promoted the analysis of gene expression. Microarrays have been applied to many areas of research, such as the comparison of normal tissues and tumor tissues,[18)] the

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classification of breast carcinomas,[19] gene expression analysis of hematopoietic stem cells,[20] and the analysis of UV response genes.[21] Exhaustive gene expression analysis after heat shock treatment is possible using microarrays. In the present study, we focused on the identification of a gene that plays an important role in the heat shock response and that is likely to be relevant to the enhancement of the hyperthermic effect.

Materials and Methods

Cell culture and hyperthermia treatment. HeLa cells were cultured at 37°C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, GIBCO-BRL Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum, 100 U/ml penicillin G and 0.1 mg/ml streptomycin. For heat shock experiments, cells (1.5×10^6) were seeded in a culture dish, which was then wrapped with hermetic film and incubated at 37°C for 24 h. The dishes were next immersed in a water bath at 44°C (±0.05°C) for 1 h. For gene expression profiling, RNA was isolated from cells 0, 3, 6, and 12 h after heat shock. Control cells were collected just prior to the heat shock treatment. The gene expression experiment was repeated twice.

RNA isolation, labeling, and hybridization. Total RNA isolation was performed using RNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. The amount of total RNA was measured with a spectrophotometer, and its quality was checked by agarose gel electrophoresis. The cDNA probes were prepared from 40 μg of total RNA using a Fluorescent Direct Label Kit (Agilent Technologies, Palo Alto, CA). Control RNA and heated sample RNA were labeled with Cy3-dCTP or Cy5-dCTP according to the manufacturer's instructions. Labeled probes were hybridized to a Human 1 cDNA microarray (No. G4100A; Agilent Technologies) containing 12 814 clones. The hybridization and washing were carried out according to the Agilent technical manual. The intensity of each hybridization signal was scanned using an Affymetrix 418 Array Scanner (Affymetrix, Inc., Santa Clara, CA).

Data analysis. Signal intensities of Cy3 and Cy5 from the 12 814 spots were quantified and analyzed using GenePix (Axon Instruments, Foster City, CA). Spots in which more than 60% of the pixels showed an intensity less than the threshold value (defined by the sum of the average intensity of the background pixel and one SD) were eliminated. The remaining signals were normalized so that the median of all signal ratios (Cy3/Cy5) would be 1.0.

Clustering by fuzzy adaptive resonance theory (fuzzy ART). Gene expression data were clustered by fuzzy ART, a method developed in our laboratory and previously described.[22] This method enables the compilation of multiple time course-gene expression patterns. In the previous paper,[22] we showed that fuzzy ART is more robust and accurate than other clustering methods. In this clustering step, we selected significantly expressed genes that showed a Cy3/Cy5 signal ratio >2.0 or <0.5 in two independent experiments. To simplify the expression patterns, the raw data that lay between 0.5 and 2.0 were converted to 0 to be used as the input data for the fuzzy operator. The vigilance parameter and recording rate were set to be 0.86 and 0.005, respectively. We also clustered gene expression data using other methods, such as hierarchical clustering,[23] k-means clustering,[24] and self-organizing map (SOM).[25] These methods were performed according to the instructions on the web site (http://rana.lbl.gov/EisenSoftware.htm).

Immunoassay. We used a human matrix metalloproteinase-3 (MMP-3) immunoassay (R&D Systems, Inc., Minneapolis, MN) for the quantitative determination of total MMP-3 in cell culture supernatants. As described above, culture dishes containing HeLa cells were immersed in a water bath at 44°C for 1 h. The cell culture supernatant was isolated from cells at 6, 12 and 24 h after heat shock. The assay was performed according to the manufacturer's instructions, and the absorbance was read at 540 nm.

Hyperthermia in the presence of MMP inhibitors (MMPI). We used the following sulfonamide-based hydroxamic acid MMP-3 inhibitors: N-isobutyl-N-(4-methoxyphenylsulfonyl)-glycylhydroxamic acid (NNGH, Fig. 4A),[26] purchased from Calbiochem-Novobiochem (San Diego, CA) and N-hydroxy-2(*R*)-[[4-methoxysulfonyl](3-picolyl)amino]-3-methylbutaneamide hydrochloride (MMI270, Fig. 4B), donated by Novartis Phama AG (Basel, Switzerland).

HeLa cells (2.4×10^4) were seeded in a 24-well plate and incubated at 37°C for 24 h. NNGH or MMI270 was dissolved in dimethylsulfoxide (DMSO) and added to the medium at the indicated concentrations 1 h before heat shock treatment. The final concentration of DMSO in the culture medium was 0.41% (v/v). The same concentration of DMSO was used as a control. Twenty-four-well plates were immersed in a water bath at 44°C for 60, 75, and 90 min. Surviving cells were counted by trypan blue dye exclusion after 3 days.

Results

Determination of heat shock conditions. HeLa cells were heat-shocked for 1 h at 43, 44 and 45°C. When the cells were treated at 43°C, the cell growth rate decreased slightly, after which the cell viability recovered, returning to the control level 24 h after heating. In contrast, cell growth was significantly suppressed at 44°C and 45°C. The cell number was approximately 75% of the initial value after 3 days at 44°C and 30% after 3 days at 45°C (Fig. 1). It was difficult to extract RNA from the cells treated at 45°C because most of the cells died. Therefore, we selected the 44°C treatment for further investigation of gene expression after heat shock treatment. Because the quality of the RNA is critical for microarray analysis, the extracted RNA was analyzed by agarose gel electrophoresis before microarray analysis to assure that the 28S and 18S ribosomal RNA bands were present clearly and that degradation had not occurred (data not shown).

Analysis of gene expression profiles. Prior to the DNA microarray analysis of heat shock samples, we assessed the reproducibility of the microarray system. Using duplicate control sample RNA (without heat shock treatment), the expression ratio is expected to be 1.0 if the two experiments are perfectly reproducible. In fact, we found that 99.14% spots showed an expression ratio between 0.5 and 2.0 (data not shown). Therefore, we deter-

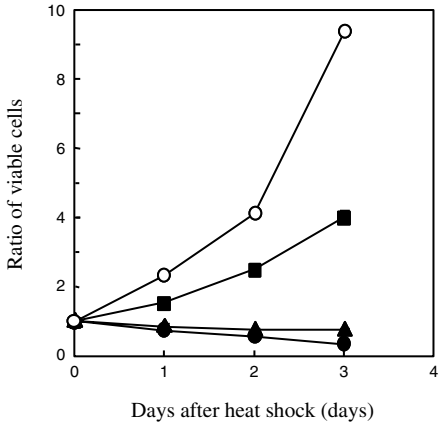

Fig. 1. Time course of cell growth after heat shock. The ratio of cell viability is based on the initial cell concentration. ○ no heat treatment (37°C), ■ 43°C, ▲ 44°C, ● 45°C.

mined that the threshold values of the expression ratios are less than 0.5 and more than 2.0.

We next examined the expression of 12 814 genes at 0, 6, 12, and 24 h. Among them, the 664 genes were beyond the threshold value in any of 4 samples. Many of these selected genes have well known functions in growth arrest/repair, DNA binding, heat shock, and other stress responses. In addition, many are transcription factors, including *hsp40*, *hsp70*, *hsp90*, *gadd45* (growth arrest and DNA damage inducible gene 45), *egr-1* (early growth response 1), *c-jun*, and *c-fos*.

We next subjected the 664 genes to fuzzy ART[22] to find the trigger gene for heat shock response. Fuzzy ART can classify the genes based on the time course of expression. In our previous studies[22] on the analysis of gene expression profiling during spore formation by the yeast *Saccharomyces cerevisiae*, fuzzy ART was able to classify 45 genes into 5 clusters. The genes involved in each cluster showed more similar expression patterns, compared with other clustering methods, such as k-means or SOMs algorithm. By applying fuzzy ART clustering, the 664 genes were classified into 7 clusters (Fig. 2) with characteristic expression patterns. In Fig. 2, weight vectors[22] acquired by fuzzy ART describe the typical pattern of each cluster. Cluster 1 consisted of 6 genes, which were strongly expressed and early responders to heat shock. Just after heat shock, there was an approximately 4-fold increase in their expression. This reached more than 20-fold increase at 3 h and then decreased to baseline within 12 h. Similar to cluster 1, cluster 2 consisted of 35 early response genes. The weight vector of cluster 2 signifies a 2-fold increase in gene expression that was maintained for several hours after heat shock. Cluster 3 genes started to be expressed within a few hours, while cluster 4 genes were expressed after 12 h. Finally, there were three clusters of suppressed genes (Fig. 2).

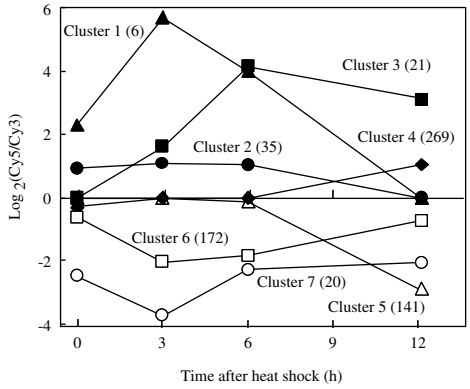

Fig. 2. Expression patterns analyzed by fuzzy ART clustering. For microarray analysis, control RNA without heat treatment and heated sample RNA were labeled with Cy3-dCTP or Cy5-dCTP. Ratios of the signal intensities are plotted against time after heat shock. These data were subjected to fuzzy ART clustering. Expression patterns obtained from fuzzy ART are indicated in each cluster. Figures in parenthesis show the number of genes included in each cluster. ▲ cluster 1 (6), ● cluster 2 (35), ■ cluster 3 (21), ◆ cluster 4 (269), △ cluster 5 (141), □ cluster 6 (172), ○ cluster 7 (20).

Other clustering methods were also employed, including hierarchical clustering, k-means clustering, and SOM. Although the 664 genes were classified into 7 clusters using these three methods, up-regulated genes could not be classified well. For example, some genes that were classified into clusters 1, 2 and 3 by fuzzy ART were placed in a single cluster by these other methods.

Genes showing enhanced expression. From the fuzzy ART analysis, cluster 1 contained 6 genes, while cluster 2 contained 35 genes. Five genes from cluster 1 and nine of the genes from cluster 2 are listed in Table 1. These are early response genes, which may play an important role in the heat shock response, for example in the repair of injured cells. Indeed, *c-fos*, *c-jun* and *egr-1*, which are well known stress response transcription factors, were included in these clusters.[27,28] Activator protein-1 (AP-1), which is a complex of Fos and Jun, is an important transcription factor for the stress response.[27] In addition, Guo *et al*. reported that *c-fos* and *c-jun* expression increased significantly within 30 min after UV irradiation.[29] Also, *fosB* is one of the Fos family transcriptional factors. Further, *egr-1* is a cell growth regulator that suppresses transformation via the expression of *TGFβ-1* (transforming growth factor β-1), and stimulates apoptosis by *trans*-activation of the *p53* gene.[30] Chuang *et al*. reported that *egr-1* is immediately expressed after oxidative stress.[31] Uracil DNA glycosylase 2 is a DNA repair enzyme that catalyzes the hydrolysis of premutagenic uracil residues from single-stranded or duplex DNA.[32] *RGS16* (regulator of G-protein signalling 16) is a novel *p53* target gene that is induced in response to genotoxic stress.[33] Induction of *RGS16* may mediate p53 regulation of signaling through G-protein coupled growth and survival factors receptors. Finally, two more of the cluster 2 genes, *hsp70* and *ppm1d* (protein phosphatase 1D magnesium-dependent, delta isoform),[34] are known stress response genes.

Because the function of these genes in stress responses has been well characterized, other genes were investigated for the selection of a candidate gene for cancer therapy by hyperthermia. We focused on MMP-3 because it has already been recog-

Table 1. A part of genes in cluster 1 and 2

GenBank	Gene name	Cluster	Expression ratio			
			0 h	3 h	6 h	12 h
L49169	FBJ murine osteosarcoma viral oncogene homolog B (FosB)	1	1.56	13.92	28.28	1.00
U70426	regulator of G-protein signalling 16	1	2.69	10.24	11.70	1.00
AA291356	uracil-DNA glycosylase 2	1	4.41	29.00	9.11	1.69
V01512	v-fos FBJ murine osteosarcoma viral oncogene homolog (c-Fos)	1	5.22	54.70	15.90	2.22
J04111	v-jun avian sarcoma virus 17 oncogene homolog (c-Jun)	1	5.55	25.14	34.29	8.46
U40992	DnaJ-like heat shock protein 40	2	1.38	2.36	2.90	1.75
AA399119	early growth response 1	2	0.95	4.51	3.40	1.30
U56725	heat shock 70 kD protein 2	2	1.36	2.97	3.20	1.77
L23808	matrix metalloproteinase 12 (macrophage elastase)	2	2.29	2.38	2.31	2.01
U78045	matrix metalloproteinase 3 (stromelysin 1, progelatinase)	2	2.53	2.49	2.59	2.12
NM_003620	protein phosphatase 1D magnesium-dependent, delta isoform	2	1.41	2.28	3.94	2.26
AA564880	ribosomal protein S24	2	2.10	1.99	1.61	1.54
AA535571	ribosomal protein S29	2	2.22	2.29	2.28	1.85
AA936430	U2 small nuclear ribonucleoprotein auxiliary factor (65 kD)	2	2.20	2.32	2.50	1.95

nized as a molecular target for cancer therapy and because inhibitors have been already developed.[35] Based on our microarray data, the *MMP-3* gene is expressed immediately after heat shock treatment. Thus, MMP-3 could be a target for cancer therapy by hyperthermia.

Induced expression of MMP-3 protein. We found that the *MMP-3* expression ratio increased 2.5-fold at 0, 3, and 6 h after heat stress (Fig. 3A). To verify that the MMP-3 protein was expressed, we examined the MMP-3 protein level in culture medium at 6, 12, and 24 h after heat shock treatment (Fig. 3B). We found that MMP-3 protein expression increased 12 h after heat shock, and that the concentration of MMP-3 was approximately 60-fold higher after 24 h of heat shock than that at the 0 h time point.

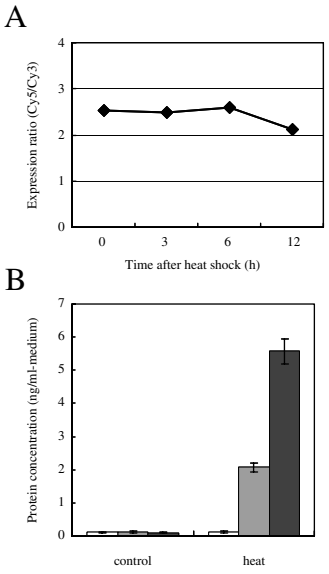

Fig. 3. Induction of MMP-3 expression. The expression ratio of the *MMP-3* gene from the microarray data (A) and the concentration of MMP-3 protein in the medium (B) were examined after heat shock or without heat shock (control). The concentrations of MMP-3 protein are indicated after 6 h (white bar), 12 h (gray bar) and 24 h (black bar) after heat shock.

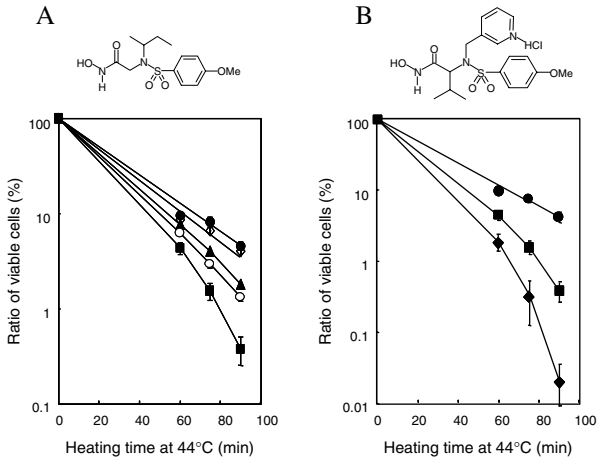

Fig. 4. Surviving cell number 3 days after heat shock treatment in the presence of MMP inhibitors, NNGH (A) and MMI270 (B). Heat shock treatment at 44°C was performed in the presence of NNGH at the concentration of 0 (●), 1.3 (◇), 6.5 (▲), 8.0 (○), 13 μM (■) or MMI270 at 13 μM (◆). In the cell viability measurement, the cell concentration at 3 days after heat shock in the control experiment without NNGH or MMI270 was taken as 100%.

Hyperthermia with MMP-3 inhibitors. Several MMPIs are commercially available. We first selected NNGH, because it is one of the most potent MMPIs available. We also used MMI270 (K_i=43 nM), which is 40-fold more potent than NNGH (K_i=1820 nM).[26]

We examined the effect of these MMP-3 inhibitors on HeLa cell viability *in vitro* after heat shock. MMPIs are generally regarded as cytostatic drugs that inhibit tumor growth but do not induce tumor regression.[36] However, we found a significant enhancement of cell death with both MMPIs (Fig. 4). In the presence of 13 μM NNGH, only 0.38% of the cells remained alive after a 90 min heat shock at 44°C. This corresponded to an approximately 12-fold increase in cell death compared with the control (no inhibitor). The enhancement of cell death was dose-dependent. In the presence of 13 μM MMI270, cell viability was only 0.02% following a 90 min heat shock at 44°C. Thus, MMI270 was approximately 20-fold more effective than NNGH. These results suggest that MMP-3 plays an important role in the repair of injured cells after heat shock and that MMP-3 inhibitors can promote heat shock-induced cell death.

We further investigated the effects of MMPIs on the heat shock treatment response in U251-SP (human glioma cell line) cells, in which *MMP-3* expression is induced after heat shock. We found that the MMPIs were able to enhance heat shock-induced U251-SP cell death, as found in HeLa cells (data not shown). This suggests that the enhancement of heat shock stress by MMPI is a general feature of MMP-expressing cells.

Discussion

In the current studies, we used microarray analysis to examine the gene expression profile during the response to heat shock. We found that the expression levels of 664 genes increased or decreased. It is well known that many *HSP* genes such as *hsp40*, *hsp60*, *hsp70*, *hsp90* and *hsp105* are expressed after heat shock to prevent protein denaturation and aggregation.[37] Many stress-regulated genes were also included in the 664 genes. Cluster 1, which was composed of genes strongly expressed just after heat shock treatment, included *c-fos* and *c-jun*. In addition, the stress response gene *egr-1* was included in cluster 2, in which the genes were early responders to heat shock and expression was maintained for several hours after heat shock. Similarly, others' reports show that the expressions of *c-fos* and *c-jun* are induced within 30 min after irradiation,[29] and the expression of *egr-1* is induced immediately after oxidative stress.[31] These results are consistent with the fact that these genes were induced by heat shock treatment and support a role for these genes in the cellular biochemical response to this stress. For example, it is likely that *egr-1* contributes to post-heat shock growth arrest and apoptosis via transcriptional regulation.

MMP-3 is a member of the MMP family. MMPs are enzymes that degrade most components of the extracellular matrix, including collagens, laminins, fibronectins, elastins, and the protein core of proteoglycans. Currently, more than 20 different MMPs have been identified and characterized. MMPs are now known to contribute to multiple steps of tumor progression and invasion, including tumor promotion, angiogenesis, and the establishment and growth of metastatic lesions in distant organ sites. Accordingly, MMPs have received much attention as molecular targets of cancer therapy. Many of the *MMP* genes are induced by growth factors, cytokines, chemical agents (e.g. phorbol esters, actin stress fiber-disrupting drugs), physical stress, or oncogenic cellular transformation, and enhanced MMP gene expression may be down-regulated by suppressive factors (e.g. TGF-β, retinoic acids, glucocorticoids).[38] The expressions of *MMP-1* and *MMP-3* are induced by cytokines (e.g.

IL-1 and TNF-α), growth factors (e.g. EGF, PDGF and bFGF) and the gene products of oncogenes such as Ras.[39, 40] However, there are few reports on *MMPs* expression after heat shock. Matsuzawa *et al.*[41] reported that one out of seven human astrocytoma cell lines expressed *MMP-3* following heat shock. Vance *et al.*[42] reported that heat shock (45°C, 1 h) of monolayer cultures of rabbit synovial fibroblasts increased expression of mRNA of collagenase (MMP-1) and stromelysin (MMP-3). Our finding that *MMP-3* expression is increased in HeLa and U251-SP cells following heat shock adds to the evidence that MMP-3 plays a role in the cellular response to this stress. Sato *et al.*[43] reported that heat shock at 42°C suppressed the production and gene expression of membrane type-1 MMP (*MT-MMP1*) in human fibrosarcoma HT-1080 cells. The results of *in vitro* tumor invasion assay in a Matrigel model indicated that heat shock inhibited the invasive activity of HT-1080 cells. MT-MMP1 is ProMMP-2 activator called MMP-14. In the DNA microarray used in the present study, the expression levels of the following *MT-MMP* genes can be assayed: *MMP-14* (*MT-MMP1*), *MMP-15* (*MT-MMP2*), *MMP-16* (*MT-MMP3*), *MMP-17* (*MT-MMP4*), and *MMP-24* (*MT-MMP5*). However, their expression levels showed an expression ratio between 0.5 and 2.0 after heat shock. The reason why the finding on *MT-MMP* expression in human fibrosarcoma HT-1080 cells is different from that for *MMP-3* in astrocytoma and fibroblasts remains unknown.

Currently, there are many MMPIs designed to mimic the peptide sequence recognized by MMP-3, and some have reached clinical trial.[36] These MMPIs were expected to be useful in the treatment of metastatic cancer and advanced cancer, but clinical trials of some MMPIs were abandoned because of low efficacy and skeletomuscular side effects. Nevertheless, MMPIs are still expected to show efficacy if administered in conjunction with another chemotherapeutic agent. NNGH and MMI270 are selective nonpeptide inhibitors of MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, MMP-12, MMP-13, and MMP-14. The following IC_{50} values of MMI270 have been reported: MMP-12, 3 n*M*; MMP-2, 4 n*M*; MMP-13, 5 n*M*; MMP-14 (membrane type-1 MMP), 6 n*M*; MMP-9, 7 n*M*; etc. In our microarray data, *MMP-1*, *MMP-3*, *MMP-10*, and *MMP-12* genes showed significantly increased after heat shock treatment, and it is likely that *MMP-1*, *MMP-3* and *MMP-12* induced here would be inhibited by NNGH and MMI270. Since an MMP-12 specific inhibitor was not examined here, the effect of inhibition of MMP-3 or MMP-12 on heat inducible cell death remains to be established. MMP-3 plays a central role in the activation of pro-MMP to active MMP, including MMP-1, MMP-7, MMP-8, and MMP-9.[44, 45] In the present report, *MMP-3* expression was significantly induced by heat shock, and the MMP-3 inhibitor strongly enhanced heat shock-induced cell death. HSP inhibitor I[15, 16] and inhibitors of PKC[17] were reported to reduce thermotolerance development, which resulted in the enhancement of cell death. Further experiments are needed to understand the role of MMP-3 in thermotolerance should be investigated.

In the present report, we examined two MMPIs, NNGH and MMI270, both of which significantly enhanced heat shock-induced cell death in HeLa cells. MMI270 was about 20-fold more effective at inducing cell death than NNGH. This is consistent with the low inhibition constant, K_i (43 n*M*), of MMI270, while that of NNGH is 1820 n*M*.[23] A phase I study of MMI270 in a group of 92 patients was reported by Levitt *et al.*[46] They found no myelotoxicity, although sustained plasma concentrations in excess of 4×mean IC_{50} values for the target enzymes were obtained, and 19 patients had stable disease for more than 90 days, although there were no tumor regressions. The enhancement of tumor cell death obtained here is a novel observation and strongly suggests that MMPIs should be used in combination with hyperthermia.

In summary, over 600 genes were significantly expressed in our microarray analysis. Using fuzzy ART clustering, we selected 41 genes that were expressed at the early phase of heat shock. We only focused on MMP-3 because a specific inhibitor was available. We found that the targeting of MMP-3 with MMPIs significantly enhanced hyperthermia-induced cell death. Other genes are also candidate genes for the enhancement of the curative effect of hyperthermia. If other genes are focused as target genes, RNAi should be a powerful tool for the suppression of their expression. In the present paper, it was found that a MMP-3 inhibitor significantly enhanced cell death by hyperthermia. This novel function of MMPIs was discovered as a result of gene expression profiling.

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