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Heat Shock Suppresses Membrane Type 1-Matrix Metalloproteinase Production and Progelatinase A Activation in Human Fibrosarcoma HT-1080 Cells and Thereby Inhibits Cellular Invasion

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Expression of membrane type-1 matrix metalloproteinase (MT1-MMP) is closely correlated with tumor invasiveness. We investigated the effect of hyperthermia on the production of MT1-MMP in human fibrosarcoma HT-1080 cells. Heat shock at 42°C suppressed the production and gene expression of MT1-MMP in HT-1080 cells. Heat shock-induced suppression of MT1-MMP production resulted in the inhibition of progelatinase A (proMMP-2) activation and the increased release of tissue inhibitor of metalloproteinases 2 from cell surface. In addition, *in vitro* tumor invasion assay in a Matrigel model indicated that heat shock inhibited the invasive activity of HT-1080 cells. These results suggest that heat shock preferentially suppresses the production of MT1-MMP and thereby inhibits proMMP-2 activation, events which subsequently inhibit tumor invasion. Therefore, heat shock shows an anti-invasive effect along with the known mechanism of inhibiting tumor growth.

The metastatic progression of malignant tumor requires the proteolytic degradation of extracellular matrix (ECM) components such as type IV collagen, laminin and proteoglycans. Matrix metalloproteinases (MMPs) play important roles in ECM degradation, and the expression of membrane type-1 matrix metalloproteinase (MT1-MMP) and MMP-2/gelatinase A (72-kDa type IV collagenase) has been closely correlated with tumor invasiveness both *in vivo* and *in vitro* (1–4). It is also reported that MT1-MMP expression is more predominant in human invasive breast carcinomas than MT2- and MT3-MMP (5). We demonstrated that MT1-MMP production is augmented in human squamous carcinoma A431 cells co-cultured with normal human fibroblasts and the predominant invasion of the tumor cells was observed in the co-culture (6). Furthermore, recent studies demonstrated that MT1-MMP digests ECM components such as types I and III collagen, gelatin, fibronectin, laminin and aggrecan (7, 8). It is therefore suggested that MT1-MMP is a key enzyme for tumor invasion and metastasis by activating proMMP-2 and degrading ECM *in vivo*, but there is no therapeutical evidence for the suppression of MT1-MMP production and a consequent decrease in tumor invasion and metastasis.

Exposing malignant cells to hyperthermia is one therapeutic strategy that can prevent tumor progression because heat shock inhibits tumor growth (9, 10). These lines of evidence allow us to speculate that hyperthermia could influence tumor invasion and metastasis. We herein investigated the effect of heat shock on tumor invasiveness by focussing on MT1-MMP production in human fibrosarcoma HT-1080 cells, and demonstrated that heat shock suppressed the production of MT1-MMP and the activation of proMMP-2, which caused the inhibition of tumor invasion *in vitro*.

Abbreviations: ECM, extracellular matrix; MMP, matrix metalloproteinase; MT-MMP, membrane type-MMP; TIMP-2, tissue inhibitor of metalloproteinases 2; MEM, minimum essential medium; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; ECL, enhanced chemiluminescence; RT-PCR, reverse transcriptase-polymerase chain reaction; GADPH, glyceraldehyde-3-phosphate dehydrogenase.

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MATERIALS AND METHODS

Cell culture and heat shock treatment. Human fibrosarcoma HT-1080 cells (Health Science Research Resources Bank, Osaka, Japan) were cultured in Eagle's minimum essential medium (MEM) (Life Technologies, Inc., Grand Island, NY) supplemented with 10% (v/v) fetal bovine serum (BioWhittaker, Walkersville, MD) and nonessential amino acids (Life Technologies, Inc.). After reaching confluence, the cells were treated at 42°C for up to 3 h in serum-free MEM supplemented with 0.2% (w/v) lactalbumin hydrolysate, and then incubated at 37°C for a further 24 h after replacement of the same fresh culture medium.

Western blotting for MT1-MMP and TIMP-2. After heat shock treatment, the culture medium was harvested and plasma membranes were prepared as described previously (11). The harvested culture medium (1 ml) and an aliquot (100 μg) of the plasma membranes were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with 10 and 12.5% (w/v) acrylamide gel to detect MT1-MMP and TIMP-2, respectively. The proteins separated in the gel were electrotransferred onto a nitrocellulose membrane and reacted with monoclonal antibody against MT1-MMP (2) and sheep anti-(human TIMP-2) antibody (a generous gift from Dr. H. Nagase, University of Kansas Medical Center) followed by alkaline phosphatase-conjugated rabbit anti-(mouse IgG) IgG and horseradish peroxidase-conjugated goat anti-(sheep IgG) IgG, respectively. Immunoreactive MT1-MMP and TIMP-2 were visualized with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (12), and enhanced chemiluminescence (ECL)-Western blotting detection reagents (Amersham Life Science, Tokyo, Japan), respectively. The relative amounts of MT1-MMP and TIMP-2 were quantified by densitometric scanning.

Gelatin zymography. The harvested culture medium (10 μl) was subjected to SDS-PAGE with 10% (w/v) acrylamide gel containing gelatin (0.6 mg/ml) (Difco Laboratories, Detroit, MI). The gel was washed with washing buffer (50 mM Tris-HCl (pH 7.5)/0.15 M NaCl/10 mM $CaCl_2$/1 μM $ZnCl_2$/0.1% (v/v) Triton X-100) to remove SDS and then incubated at 37°C in incubation buffer (50 mM Tris-HCl (pH 7.5)/0.15 M NaCl/10 mM $CaCl_2$/1 μM $ZnCl_2$). Thereafter the gel was stained with Coomassie brilliant blue R-250, and gelatinolytic activity was detected as unstained bands on a blue background as described (13).

Human TIMP-2 cDNA cloning. Cytoplasmic RNA in HT-1080 cells was isolated with ISOGEN (Nippon Gene Co., Toyama, Japan). One microgram of the isolated RNA was subjected to the amplification of TIMP-2 cDNA by reverse transcriptase-polymerase chain reaction (RT-PCR) as described previously (14). The TIMP-2 primers were 5′-GGAATTCCATGGGCGCCGC-3′ (sense: 263–281 bp) and 5′-TTGGAGGTCGACTTATGGGTC-3′ (antisense: 925–945 bp) (15), in which *Eco*R I and *Sal* I were designed (underlines), respectively. PCR was performed for 35 cycles at 92°C for 40 sec, at 56°C for 40 sec and 72°C for 1 min, and then the PCR product was subcloned into pGEM-T vector (Promega, Madison, WI). The cDNA sequence of TIMP-2 was confirmed with Sequenase version 2.0 DNA sequence kit (U.S. Biochemicals, Cleveland, OH) according to the manufacturer's instructions. The TIMP-2 cDNA was obtained by restriction digestion with *Eco*R I and *Sal* I and then subjected to Northern blotting.

Northern blotting for MT1-MMP and TIMP-2. Twenty micrograms of RNA was denatured and separated by electrophoresis on a 1.0% (w/v) formaldehyde-denatured agarose gel, and then transferred onto a nylon membrane (GeneScreen) (DuPont, Boston, MA). The membrane was hybridized with cDNA probes for MT1-MMP (2), TIMP-2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Clontech Laboratories, Inc., Palo Alto, CA) labeled with [α-^{32}P]dCTP (111 TBq/mmol) (DuPont NEN) with a random labeling kit (Boehringer Mannheim Biochemicals, Indianapolis, IN). The relative amounts of MT1-MMP and TIMP-2 mRNA were quantified by densitometric scanning and indicated after correction for the level of GAPDH mRNA.

Tumor invasion assay. Tumor invasion assay was performed in Matrigel Invasion Chamber (Nippon Becton Dickinson Co., Ltd., Tokyo, Japan). HT-1080 cells (4×10^4 cells) were added to the inserts in 200 μl of 0.1% (w/v) bovine serum albumin/MEM and then incubated at 37°C for 1 h to achieve cell adhesion. The cells were maintained at 37 or 42°C for 3 h and then further incubated for 24 h at 37°C. To determine whether the invasive activity of HT-1080 cells is dependent on MMP activity, the cells were maintained in the presence of recombinant human TIMP-2 (a generous gift from Dr. H. Nagase) at 37°C after the cell adhesion. The cells on the upper surface of the membrane were wiped off with a cotton swab and the cells on the lower side were fixed with 4% (w/v) formaldehyde and then stained with hematoxylin. The number of cells in ten randomly chosen areas per membrane was visually counted under a light microscope at 200-fold magnification. We confirmed that the heat shock treatment did not influence the functional property of Matrigel as a barrier because the invasive activity was unchanged when the Matrigel in the chamber was treated with heat shock for 3 h prior to seeding the cells (data not shown).

Total protein biosynthesis. Confluent HT-1080 cells in 24-multiwell plates were treated with heat shock at 42°C for 3 h in 0.2% (w/v) lactalbumin hydrolysate/MEM and then incubated in the same fresh medium containing [^{3}H]amino acid mixture (37 kBq/well) (Amersham Life Science) for a further 2 h at 37°C. Proteins in the harvested culture media were precipitated with trichloroacetic acid at a final concentration of 3.3% (w/v) and then washed three times with 3.3% (w/v) trichloroacetic acid. The cells were also washed three times with Ca^{2+}- and Mg^{2+}-free phosphate buffered saline and then fixed with cold 10% (w/v) trichloroacetic acid for 30 min. The fixed cells were washed once with ethanol:ether (3:1, v/v). The precipitated proteins from the culture media and the fixed cells were dissolved in 0.5 M NaOH. The amount of radioactivity in the extracellular and intracellular labeled proteins was measured in a liquid scintillation counter.

Statistical analysis. Data were analyzed by Student's *t*-test; $p < 0.05$ was considered to be statistically significant.

RESULTS

Heat shock suppresses MT1-MMP production and proMMP-2 activation in HT-1080 cells. We first examined the time period of heat shock to influence tumor invasiveness by monitoring proMMP-2 activation. When HT-1080 cells were treated with heat shock at 42°C for $\frac{1}{2}$ to 5 h and then incubated for a further 24 h at 37°C, the constitutive proMMP-2 activation was not influenced within 2 h (data not shown) but apparently suppressed for 3 h (Fig. 1A). Nevertheless, the total protein synthesis by monitoring [^{3}H]amino acid incorporation was not altered in the heat shocked HT-1080 cells [4.20 $\pm$ 0.05 ($\times 10^3$ dpm/well)] compared with the untreated cells [4.38 $\pm$ 0.07 ($\times 10^3$ dpm/well)]. Furthermore, the production of heat shock protein-70 was augmented by heat shock in HT-1080 cells (data not shown). These results suggest that the inhibition of proMMP-2 activation by heat shock is not due to the decrease in the cellular functions. Therefore we decided that heat shock for 3 h was the minimal and sufficient period to elicit the biological activity.

We next examined the effect of heat shock on the production of MT1-MMP in HT-1080 cells. The consti-

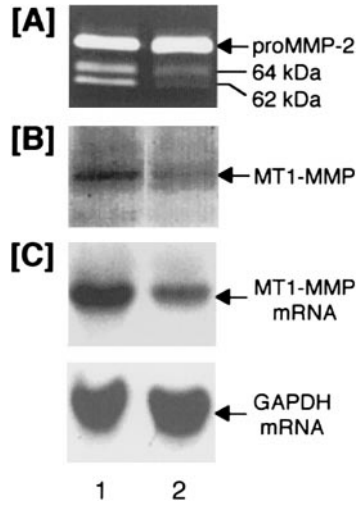

FIG. 1. Heat shock suppresses the production and gene expression of MT1-MMP and inhibits proMMP-2 activation in HT-1080 cells. Confluent HT-1080 cells were treated with heat shock at 42°C for 3 h and then incubated for a further 24 h at 37°C after replacement with fresh serum-free MEM. The harvested culture medium was subjected to gelatin zymography [A]. Plasma membranes (100 μg) and total cytoplasmic RNA (20 μg) were subjected to Western blotting [B] and Northern blotting [C], respectively. Relative amounts of steady-state MT1-MMP mRNA [C, upper panel] were quantified by densitometric scanning followed by normalizing against GAPDH mRNA [C, lower panel]. The three independent experiments were highly reproducible and typical data are shown. Lane 1, cells without heat shock; lane 2, heat-shocked cells. 64 kDa, intermediate form of MMP-2 and 62 kDa, active form of MMP-2.

tutive production and gene expression of MT1-MMP were decreased by 16% and 38% of the control, respectively, in the heat shocked HT-1080 cells (Figs. 1B and 1C, respectively). The spontaneous activation of proMMP-2 was inhibited and the level of proMMP-2 was relatively increased by heat shock (Fig. 1A), but the gene expression of proMMP-2 was not altered in the heat shocked HT-1080 cells (data not shown). The suppression of MT1-MMP gene expression and proMMP-2 activation occurred as early as 3 h and was easily detected for 6-10 h after heat shock (data not shown). These results suggest that heat shock preferentially suppresses the production of MT1-MMP and thereby the activation of proMMP-2 is inhibited in HT-1080 cells.

Heat shock increases TIMP-2 release from cell surface. Since TIMP-2 participates in the MT1-MMP-mediated proMMP-2 activation by forming MT1-MMP/TIMP-2/proMMP-2 trimeric complex (16, 17), we investigated the regulation of TIMP-2 production in the heat shocked HT-1080 cells. As shown in Fig. 2A, the extracellular level of TIMP-2 (left panel) was augmented 2-fold in the heat shocked HT-1080 cells. In contrast, TIMP-2 detected in the cell membranes (right panel) of HT-1080 cells was almost completely eliminated by the heat shock, but the steady-state level of TIMP-2 mRNA (3.5 kb) was not altered in the heat shocked HT-1080 cells (Fig. 2B), indicating that the heat shock-mediated alteration of TIMP-2 production was not due to the transcriptional modification. These results therefore suggest that the heat shock-mediated accumulation of TIMP-2 in the extracellular space is due to its augmented release from the cell membranes by decreasing the expression of MT1-MMP on cell surface as a TIMP-2 receptor (16, 17).

Heat shock inhibits in vitro invasive activity of HT-1080 cells. To investigate whether the heat shock-mediated suppression of MT1-MMP production and proMMP-2 activation could inhibit the tumor invasion, the *in vitro* invasion assay with a Matrigel model was performed. As shown in Fig. 3, the invasive activity of HT-1080 cells was decreased by heat shock (48.4 $\pm$ 9.7%, lane 2). The invasiveness of HT-1080 cells was also diminished by exogenously adding recombinant human TIMP-2 (32.9 $\pm$ 12.4%, lane 3). These results suggest that the decreased MT1-MMP production and proMMP-2 activation by heat shock result in the inhibition of tumor invasion.

DISCUSSION

MT1-MMP plays an important role in tumor invasion and metastasis by not only activating proMMP-2 but also degrading ECM components by itself *in vitro* and *in vivo* (1, 7, 8). It therefore seems that suppression of MT1-MMP production would cause the inhibition of tumor invasion. On the other hand, hyperthermia elicits to prevent tumor progression *in vivo* and *in vitro*. Urano *et al.* (9) reported that whole-body hyperthermia decreases the growth of murine tumors *in vivo*. Cellier *et al.* (10) also reported that heat shock induces the *in vitro* differentiation of leukemia U937

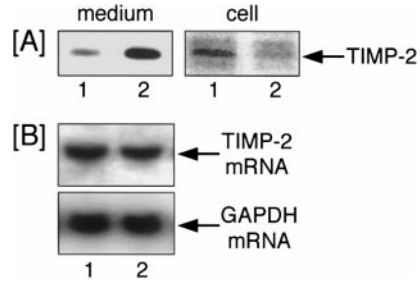

FIG. 2. Heat shock increases TIMP-2 release from the cell surface. Confluent HT-1080 cells were treated with the heat shock as described in the legend to Fig. 1. The harvested culture medium and plasma membrane (100 μg) were subjected to ECL-Western blotting for TIMP-2 [A]. Total cytoplasmic RNA (20 μg) was subjected to Northern blotting for TIMP-2 (upper panel) and GAPDH (lower panel) [B]. The three independent experiments were highly reproducible and typical data are shown. Lane 1, cells without heat shock; lane 2, heat-shocked cells.

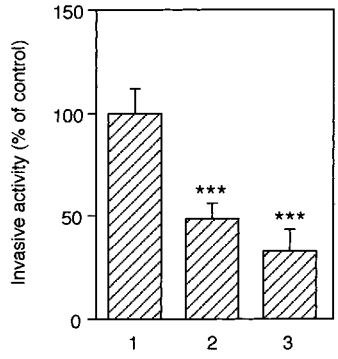

FIG. 3. Heat shock inhibits *in vitro* tumor invasion. HT-1080 cells (4×10^4 cells) were put into an insert chamber coated with Matrigel and then incubated at 37°C for 1 h to achieve cell adhesion. The cells were treated with heat shock at 42°C for 3 h and then further incubated for 24 h at 37°C. The number of invaded cells in 10 randomly chosen areas per membrane was visually counted under a light microscope at 200-fold magnification. The three independent experiments were highly reproducible and typical data are shown. Lane 1, cells without heat shock; lane 2, heat-shocked cells; lane 3, cells treated with TIMP-2 (100 ng/ml). ***, significantly different from cells without heat shock ($p < 0.001$).

cells. In the present study, we demonstrated that heat shock suppressed the production and gene expression of MT1-MMP in HT-1080 cells (Figs. 1B and 1C). The activation of proMMP-2 was also inhibited without the alteration of its gene expression in heat shocked HT-1080 cells (Fig. 1A and data not shown). These results suggest that MT1-MMP production is transcriptionally suppressed by heat shock and thereby proMMP-2 activation is inhibited in HT-1080 cells. So far, concanavalin A, phorbol ester and cell-cell or cell-matrix interaction has been reported to increase the MT1-MMP production in tumor and normal cells (6, 18–21). This is the first evidence that MT1-MMP production is efficiently and preferentially reduced by heat shock.

TIMP-2 accelerates the MT1-MMP-mediated proMMP-2 activation by forming the trimeric complex with MT1-MMP and proMMP-2 on cell surface (16, 17). Our results showed that TIMP-2 as well as MT1-MMP was detected in the cell membranes accompanied with the augmentation of proMMP-2 activation. It was of interest that the heat shock decreased the membrane-associated TIMP-2 and augmented the accumulation of extracellular TIMP-2 (Fig. 2A) without modulating the gene expression of TIMP-2 (Fig. 2B). These results strongly suggest that the heat shock-induced alteration of TIMP-2 distribution in HT-1080 cells closely contributes to the decreased production of MT1-MMP which functions as a TIMP-2 receptor on cell surface (17).

It is of interest whether the suppression of MT1-MMP production and proMMP-2 activation, and the subsequent increase in TIMP-2 release by the heat shock would be reproduced in other types of tumor cells. Similar results were obtained in heat shocked human epidermoid squamous carcinoma A431 cells and human oral squamous carcinoma SAS cells (Sato, T., Sawaji, Y., and Ito, A., unpublished data), suggesting that the suppressive action of heat shock on MT1-MMP production might be specific for tumor cells, at least in the human fibrosarcoma and squamous carcinoma cells tested.

Regarding the clinical trial for inhibiting tumor invasiveness, synthetic MMP inhibitors such as Marimastat and Batimastat have been investigated *in vitro* and *in vivo* (22). We demonstrated that heat shock decreased the invasive activity of HT-1080 cells by the mechanism in which MT1-MMP production is suppressed and proMMP-2 activation is inhibited by heat shock (Fig. 3). Our novel finding leads to the notion that heat shock can be applied as a therapeutic strategy to prevent tumor invasiveness.

Vance *et al.* (23) reported that heat shock increases the gene expression and production of proMMP-1/interstitial procollagenase and proMMP-3/prostromelysin-1 in rabbit synovial fibroblasts. They also demonstrated that putative heat shock elements are identified in the DNA sequence of rabbit proMMPs-1 and -3 promoter regions, which could interact with heat shock transcriptional factor and thereby proMMPs-1 and -3 gene expression may be augmented (23). Furthermore, Hitraya *et al.* (24) reported that heat shock augments proMMP-1 mRNA expression in human synovial fibroblasts by increasing transcriptional activity through the AP-1 binding site in proMMP-1 promoter. Since there is as yet no information on the 5′ flanking regions of MT1-MMP gene, we cannot explain in detail the mechanism of the suppression of MT1-MMP gene expression by heat shock and need to investigate it further. Nonetheless, we suggest that the heat shock-mediated regulation of MT1-MMP may be different from that of proMMPs-1 and -3 at their own promoter site.

In conclusion, we demonstrated that heat shock preferentially suppresses MT1-MMP production, and thereby inhibits proMMP-2 activation and increases the extracellular level of TIMP-2 in HT-1080 cells, which events eventually result in the inhibition of tumor invasion *in vitro*. Therefore, we suggest that heat shock actually has an anti-invasive effect along with the known mechanism of inhibiting tumor growth.

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