Methylglyoxal (MG) is one of the side-products in glycolysis, and it reacts with proteins under physiological conditions. Here, we identified heat-shock protein 27 (Hsp27) as a major MG-modified protein in cells. MG modification of Hsp27 selectively occurs at Arg-188 to form argpyrimidine, and mutation in the residue represses the formation of a large oligomer. This modification process is essential to its repressing activity for cytochrome c-mediated caspase activation. Inhibition of MG modification of Hsp27 causes sensitization of the cells to anti-tumor drug-induced apoptosis. Thus, MG is a novel modulator of cell survival by directly incorporating with the specific protein residue.

Methylglyoxal (MG), a dicarbonyl compound, is one of the side-products in glycolysis and is involved in a variety of detrimental processes, including advanced glycation endproducts (AGEs) formation with protein (1) and DNA modification (2, 3). The formation of AGEs in vivo contributes to the pathophysiology associated with aging and the long-term complications of diabetes (4). Recently, it has been shown that MG accumulates rapidly in endothelial cells cultured under hyperglycemic conditions (5). Thus, glycation of proteins by MG has been linked to mechanisms of disease.

MG is hypothesized to be a modulator of cell proliferation because of its cytotoxic properties. Indeed, the high glycolytic activity of proliferating cells, such as tumor cells, increases the intracellular levels of MG (6, 7). The principal route for MG catabolism is the glyoxalase pathway, which consists of two enzymes, glyoxalase I (GLO1) and glyoxalase II. Abnormal expression or activity of GLO1 has been demonstrated in some human tumors, including prostate and colon (8, 9). Recently, we reported that GLO1 was frequently over-expressed in human lung carcinoma cells and that the expression levels of GLO1 are closely linked with the cellular sensitivity to the GLO1-inhibitor agent (10). These observations suggest that the increase of glycolytic activity could be associated with the tumorigenicity.

The post-translational modification of protein, including phosphorylation, acetylation, methylation, and ubiquitination, is involved in the regulation of cell proliferative activity. It has been demonstrated that MG reacts with arginine and lysine residues to form N-(5-hydroxy-4,6-dimethylpyrimidin-2-yl)-1-ornithine (argpyrimidine) (11–14), N-carboxyethyllysine (15), and an MG-derived lysine-lysine cross-linking structure (imidazolysine) (12, 16–17). Argpyrimidine, one of the MG-arginine adducts, was detected in renal tissues (13) and lens proteins (15) from diabetic patients upon immunostaining and HPLC analysis. In tumor cells with high glycolytic activity, however, MG-modified proteins have not been identified nor is the functional biological significance well defined.

Here, we found that the formation of argpyrimidine in some proteins was constitutively detected in several human cancer cell lines. To clarify proteins that are regulated by MG, we purified MG-modified protein by immunoaffinity chromatography using an anti-argpyrimidine antibody. We found that MG modified heat-shock protein 27 (Hsp27) at the C-terminal Arg-188 in cells. We demonstrated that MG modification was involved in Hsp27 oligomerization to prevent cytochrome c-mediated caspase activation. These observations indicated that the post-translational modification by MG plays an important role in regulating Hsp27-mediated cell survival.

EXPERIMENTAL PROCEDURES
Reagents and Cell Culture—Argpyrimidine was kindly provided by the NOF Corporation (Tokyo, Japan). [2-14C]MG was custom synthesized by Muromachi Pharmaceuticals Co. (Tokyo, Japan). Camptothecin (CPT) was purchased from Wako Pure Chemical Industries (Osaka, Japan), and etoposide (VP-16) was kindly provided by Bristol-Myers Squibb Co. Ltd. OPB-9195 was developed by Otsuka Pharmaceuticals (Tokyo, Japan). Human leukemia U937, K562, HL-60, Jurkat, lung cancer NCI-H23, A549, colon cancer HT-29, renal cancer ACHN, and prostate cancer PC-3 cells were cultured in RPMI 1640 medium supplemented with 5% fetal bovine serum. 293T and HT1080 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum.

Affinity Purification of MG-Modified Protein—U937 cells (106 cells) were lysed in buffer A (50 mM Hepes-KOH, pH 7.0, 0.1% CHAPS, 2 mM EDTA, 10 mM dithiothreitol, 10% glycerol) by Dounce homogenization. The lysate was clarified through successive centrifugation steps (a 1,000 × g spin followed by a 10,000 × g spin) at 4 °C. Mouse monoclonal anti-argpyrimidine antibody (mAb3C) (NOF Corporation) was coupled with Affi Gel-HZ (Bio-Rad), according to the supplier’s instructions. The cell lysate was applied to an immunoaffinity column, washed with buffer A, and then eluted with a buffer containing 0.1 M glycine-HCl, pH 2.5.
ity column equilibrated with buffer A. The column was washed with buffer A containing 0.5 M NaCl to remove unbound proteins. Bound proteins were eluted with 0.2 M glycine-HCl (pH 2.5). Column fractions were resolved on SDS-PAGE, and proteins were visualized using Coomassie Blue staining.

**Amino Acid Sequence Analysis**—Purified 27-kDa protein was digested with lysyl endopeptidase from Lysozyme enzyme (Roche Molecular Biochemicals) in 50 mM Tris/HCl, pH 8.5, at 37 °C for 16 h. The peptide fragments obtained were separated by reversed-phase HPLC on a column of Purosil C18 (Waters, Milford, MA) using linear gradient (0 to 60%) of 2-propanol/acetonitrile (7:3) in distilled water containing 0.1% trifluoroacetic acid at a flow rate of 1 ml/min. Elution profiles were monitored by absorbance at 220 nm, and the peptide fragments were manually collected. The amino acid sequence analyses of the peptide fragments fractionated by HPLC were performed with PPSQ-21 gas-phase sequencer (Shimadzu, Kyoto, Japan).

**Flow-cytometric Analysis**—The wild-type human Hsp27 gene and its mutants, in which arginine residues were substituted with glycine residues, were inserted into the pFLAG-CMV-1 vector (Kodak, New Heaven, CT) or pcDNA3 (Invitrogen). Mutagenesis of Hsp27 cDNA was performed using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Transient transfection of 293T cells was performed by using the calcium phosphate precipitate method. Stable transfection of HT1080 cells was performed by using the FuGENE 6 transfection reagent (Roche Molecular Biochemicals), and transfected cells were treated with 800 µg/ml G418 (Invitrogen). Expression of Hsp27 in isolated clones was analyzed using immunoblot analysis.

**Identification of MG-modified proteins**—We detected argpyrimidine, an MG-arginine adduct, in human cancer cells by immunoaffinity chromatography using anti-arginine antibody. The antibodies used were anti-argpyrimidine mAb, anti-Hsp70 mAb (StressGen), and anti-cleaved caspase-9 and -3 (Cell Signaling Technology). For immunostaining analysis, cells were fixed on a Lab-Tec II chamber slide (Nunc) with cold methanol and blocked with 1% bovine serum albumin in phosphate-buffered saline. The fixed cells were incubated with an anti-argpyrimidine antibody. The cells were then washed with phosphate-buffered saline and incubated with anti-mouse IgG-fluorescein isothiocyanate (Santa Cruz Biotechnology, Inc.). The cells were analyzed using fluorescence microscopy.

**RESULTS**

**Identification of MG-modified proteins**—We detected argpyrimidine, an MG-arginine adduct, in human cancer cells by immunoaffinity chromatography using anti-arginine antibody. The antibodies used were anti-argpyrimidine mAb, anti-Hsp70 mAb (StressGen), and anti-cleaved caspase-9 and -3 (Cell Signaling Technology). For immunostaining analysis, cells were fixed on a Lab-Tec II chamber slide (Nunc) with cold methanol and blocked with 1% bovine serum albumin in phosphate-buffered saline. The fixed cells were incubated with an anti-argpyrimidine antibody. The cells were then washed with phosphate-buffered saline and incubated with anti-mouse IgG-fluorescein isothiocyanate (Santa Cruz Biotechnology, Inc.). The cells were analyzed using fluorescence microscopy.

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The human Hsp27 protein contains 16 arginine residues. To identify the MG modification site of Hsp27, all arginine codons were converted to glycine codons, and 293T cells were transfected with mock, FLAG-tagged Hsp27, or green fluorescent protein (GFP). After transfection for 4 h, cells were treated with 100 μM [14C]MG for 40 h. Cell lysates were then immunoprecipitated with anti-FLAG, and the bound fractions were analyzed by measuring radioactivity. B, U937 cell lysates were immunoprecipitated with either anti-Hsp27 or anti-JNK1 (negative control), and the bound fractions were analyzed using anti-argpyrimidine Western blot analysis. C, 293T cells were transfected with mock, FLAG-tagged Hsp27, or the indicated mutants. The cell lysates were immunoblotted with the indicated antibodies. Molecular size markers are indicated (in kDa). D, 293T cells were transfected with mock, FLAG-tagged Hsp27, or the indicated mutant and determined as in A.

The 27-kDa protein it was digested by lysyl endopeptidase and resolved as individual peptides by HPLC. As a result the internal peptides identified with human Hsp27 upon comparison with standard sequencing databases in the public domain (BLAST) (Fig. 1E).

Modification of Hsp27 by MG in Cells—To determine whether MG could bind directly to Hsp27, we assessed the [14C]MG in FLAG-tagged Hsp27 incorporation into 293T cells. The immunoprecipitated Hsp27 showed an ∼5-fold increase in MG binding over the control or the immunoprecipitated green fluorescent protein (Fig. 2A). To confirm modification of Hsp27 by MG, we performed immunoblot analysis after immunoprecipitation with the anti-Hsp27 antibody. As shown in Fig. 2B, we found that endogenous Hsp27 formed argpyrimidine in U937 cells. These results indicated that Hsp27 was modified by MG to form argpyrimidine.

The human Hsp27 protein contains 16 arginine residues. To identify the MG modification site of Hsp27, all arginine codons in Hsp27 cDNA were converted to glycine codons, and 293T cells were transfected with those mutants. As shown in Fig. 2C, only the mutation of Hsp27 at arginine 188 to glycine (Hsp27/R188G) significantly decreased the formation of argpyrimidine. A similar result was obtained with the lysine mutant Hsp27/R188K (data not shown). Consistently, Hsp27/R188G did not interact with [14C]MG in cells (Fig. 2D). These results indicate that MG specifically modified Hsp27 at the C-terminal Arg-188 to form argpyrimidine in cells.

Involvement of MG Modification in Hsp27 Oligomerization—Next, we stably transfected HT1080 cells with the empty pcDNA3 plasmid (mock) or the plasmid containing wild-type or the R188G-mutated Hsp27 cDNA. Expression of these proteins was determined by immunoblot analysis (Fig. 3A). The doubling time of the HT1080 cells was not affected by the overexpression of these proteins.

FIG. 3. Involvement of MG modification in Hsp27 multimerization. A, HT1080 cells were stably transfected with mock, pcDNA3-Hsp27, and R188G mutant. The expression of Hsp27 or argpyrimidine was analyzed using immunoblot analysis. B, the Hsp27 (WT) or mutant (R188G) extracts were fractionated by size distribution gel filtration. Hsp27 concentration was determined in each fraction by Western blot analysis. The apparent size (in kDa) of gel filtration markers is indicated. C, argpyrimidine of fraction 14 from WT in B was analyzed using immunoblot analysis.

It was previously (19, 20) reported that human Hsp27 could form oligomeric complexes resulting in both smaller and larger oligomers. We examined the effect of MG modification on Hsp27 oligomerization. The oligomeric size of Hsp27 in cells was analyzed by size distribution gel filtration chromatography. The wild-type Hsp27 formed complexes of 50–650 kDa with both smaller and larger oligomers (Fig. 3B, upper panel). The fraction containing large oligomeric Hsp27 was recognized by the anti-argpyrimidine antibody (Fig. 3C). In contrast, the R188G mutant exclusively formed small multimers lower than 100 kDa (Fig. 3B, lower panels). These results suggest that MG modification could be involved in the formation of high-order molecular complexes of Hsp27.

Involvement of MG modification in Hsp27 multimerization—To examine the effect of MG modification on apoptosis induction, cells were treated with several anti-tumor agents. The wild-type Hsp27 transfected cells were significantly resistant to CPT and etoposide (VP-16) compared with the parental or mock-transfected cells (Fig. 5A). The antiapoptotic activity of Hsp27 was abrogated by the MG-binding site mutant R188G. Unexpectedly, R188G-mutated Hsp27...
Cell-free extracts were treated with 10 μM cytochrome c and 1 mM dATP for 30 min. Caspase-3- and -9-like activity were assessed by measuring the cleavage of DEVD-MCA and LEHD-MCA substrates, respectively. The vertical bars represent S.D. values of triplicate determinations. 

**Fig. 4. Modification of Hsp27 by MG is required for preventing cytochrome c-mediated caspase activation.** A. 293T cells were transfected with mock, FLAG-tagged Hsp27, or the R188G mutant. Cell-free extracts were treated with 10 μM cytochrome c and 1 mM dATP for 30 min. Caspase-3- and -9-like activity were assessed by measuring the cleavage of DEVD-MCA and LEHD-MCA substrates, respectively. The equal amounts of cell-free extracts were incubated with 10 μM cytochrome c and 1 mM dATP for the indicated times, and caspase processing was assayed using immunoblot analysis.

Transflectant cells were markedly sensitized against these agents (Fig. 5A). Consistent with this, CPT- and VP-16-induced caspase activation was partially inhibited in the wild-type Hsp27 transflectant cells and was enhanced in the R188G-mutated Hsp27 transflectant cells (Fig. 5B).

To define the role of endogenously produced MG further, we examined the effect of an inhibitor of advanced glycation, OPB-9195, on anti-tumor drug-induced cell death. OPB-9195 has been shown to trap glucose-derived reactive carbonyl compounds, such as MG, glyoxal, and 3-deoxyglucosone, and thereby to inhibit the formation of AGEs (21, 22). In wild-type Hsp27 transflectant cells, OPB-9195 markedly enhanced VP-16-induced apoptosis but was less effective in the mock-transfected cells (Fig. 5C). Moreover, to clarify the link between MG modification of Hsp27 and the effect of endogenous MG on apoptosis, we did the same experiments using Hsp27-positive and -negative leukemia cells. OPB-9195 significantly accelerated apoptosis in U937 cells that expressed large amounts of Hsp27 but not in Jurkat and HEL cells that did not express Hsp27 protein (Fig. 5, D and E). In U937 cells, removal of intracellular MG by lengthy treatment with OPB-9195 resulted in reduced MG modification of Hsp27 (Fig. 5F) and provoked a change in the size distribution of the complexes that shifted from large oligomers to small oligomers (Fig. 5G). These results indicated that MG modification of Hsp27 is essential for the protective ability against apoptosis induced by anti-tumor agents.

**DISCUSSION**

Endogenously produced MG plays roles in apoptosis (23–25), growth inhibition (25), mutagenesis (3), and AGE formation (27, 28). Recently, it was shown that high levels of MG are present in cultured cells, and the most biogenic MG (>99%) is involved in reversible and irreversible interactions in vivo (29). However, the full molecular targets of MG remain unknown. In this study, we identified proteins that are modified by MG in human carcinoma cells, suggesting a novel mode for regulation of cell death. We indicated that MG modified endogenous or over-expressed Hsp27 to form argpyrimidine, one of the MG-adducts (Fig. 2, A and B). MG modification of Hsp27 could be of general significance because argpyrimidine was detected in several cell lines (Fig. 1C) and tissues (data not shown). MG specifically modified Hsp27 only at Arg-188 in cells. Thus, it is probable that this modification is mediated by an unidentified enzymatic reaction. Also, MG may modify cellular proteins other than Hsp27. Indeed, we observed some polypeptides that formed argpyrimidine by immunopurification (Fig. 1B). Moreover, in addition to arginine, lysine residues of proteins are potential targets of MG. It has been reported that MG forms N-carboxyethyllysine (15), an MG-adduct, in tissue proteins in aging and age-related diseases. These observations suggest that endogenous MG could physiologically modify cellular proteins, including Hsp27.

Hsp27 is expressed at various levels in different cell types and tissues. In cells, Hsp27 exists as homotypic multimers ranging from dimers up to 700-kDa oligomers (19, 20). Hsp27 is regulated at post-translational levels by stress and by cytokines and growth factors that trigger stress-activated protein kinase 2 (SAPK2/p38), the upstream activator of MAPKAP kinase 2 (30–32). Phosphorylation causes a major change in the structure of Hsp27, a shift from large 700-kDa multimers to dimers (19, 20). We examined the phosphorylations of Hsp27 using phospho-specific antibodies. In the R188G mutant of Hsp27, Hsp27 phosphorylation was not elevated but was rather decreased, compared with wild-type Hsp27. These observations indicate that MG modification contributes to the formation of high-order molecular complexes of Hsp27 through a mechanism that is independent of phosphorylation. Recently, the structure of a small Hsp from Methanococcus jannaschii, Hsp16.5, was defined by crystallographic analysis (33). Hsp16.5 forms a homogenous multimer comprising 24 monomers (33, 34). Each subunit in a 2-fold related dimer makes contact with other subunits in the complex. The C-terminal region of one subunit reaches out and interacts with neighboring subunits by hydrophobic interaction and backbone hydrogen bonding (33). In Hsp27, our results show that the Arg-188 residue at the C terminus is modified by MG to form argpyrimidine. Further, MG modification of the C terminus could be involved in hydrophobic interaction with neighboring subunits of Hsp27 and the formation of large oligomers because argpyrimidine has the aromatic hydroxy group, just as the tyrosine residue does. Further studies are needed to clarify the structural organization of Hsp27 in the process of MG modification.

Over-expression of Hsp27 enhances the survival of cells exposed to a variety of death stimuli, including oxidative stress (20), tumor necrosis factor-α (TNF-α) (20, 35), and anti-tumor agents (18, 36). Various mechanisms for its protective activity have been proposed. Hsp27 was reported to inhibit the processing of procaspase-9 and the other downstream enzymes (37, 38). Our present results show that MG modification of Hsp27 is essential to prevent caspase-9 and -3 activation (Fig. 4). It has been reported that Hsp27 binds to cytochrome c and prevents cytochrome c-mediated caspase activation (37). Moreover, the protective activity of Hsp27 is correlated with the ability of the protein to form large oligomers (20). Consistent with these observations, in the present study, the wild-type Hsp27 proteins that can form large oligomers interact with cytochrome c.

**H. Sakamoto, T. Mashima, K. Yamamoto, and T. Tsuruo, unpublished observation.**
to interfere with caspase-dependent apoptosis (Fig. 4 and data not shown). Conversely, Hsp27/R188G that cannot form the large oligomers did not. Therefore, MG modification may modulate the protective ability of Hsp27 through the interaction of cytochrome c.

We speculate that the protective effect of transfected Hsp27 is not strong in HT1080 cells because HT1080 cells already express some amounts of Hsp27 under constitutive conditions (Fig. 3A). Supporting this notion was the observation of a cytoprotective effect of Hsp27 in Jurkat cells, which do not express Hsp27 (data not shown). As for the pro-apoptotic effect of R188G, we have data that shows, when HT1080 cells were transiently transfected with plasmids expressing FLAG-Hsp27 (WT) plus R188G, addition of R188G caused dissociation of high molecular weight FLAG-Hsp27 oligomers to lower molecular-sized complexes (data not shown). When R188G was co-expressed with Hsp27 (WT) in Jurkat cells that did not express endogenous Hsp27, the cytoprotective effect of Hsp27 (WT) against VP-16-induced apoptosis was abolished. On the other hand, when R188G was expressed alone, we did not observe enhancement of apoptosis (data not shown). These results indicate that the R188G mutant acts as a dominant negative inhibitor of endogenous Hsp27. In addition, besides cytochrome c, Hsp27 can interact with such apoptosis-related proteins as protein kinase B (PKB/Akt) (39), which generates a survival signal in response to growth factor stimulation, and Daxx (40), a mediator of Fas-induced apoptosis, and can regulate the protective function against apoptotic cell death. MG modification of Hsp27 may also affect the interaction between Hsp27 and such proteins as these to modulate apoptosis at a step upstream of caspase activation. Regulation of MG-modified Hsp27 is of great interest and could provide further insights into the protective ability of Hsp27.

To further determine the chaperone activity of WT or R188G mutants in cells, we estimated the protective activity of each protein against heat inactivation of luciferase in transiently transfected HT1080 cells using the method previously described (41). In short, cells were subjected to heat treatment at 45 °C for 6 h and then cultured at 37 °C for 16 h. The wild-type Hsp27 showed a strong protective effect against heat inactivation of luciferase, whereas that effect was greatly reduced in the R188G mutant of Hsp27 (data not shown). These data indicate that MG modification plays a critical role in chaperone activity of Hsp27.

Homeostasis is maintained through a balance between cell proliferation and cell death. MG can alter the balance because it induces or inhibits apoptosis. High concentrations of MG act as a pro-apoptotic modulator by activating c-Jun N-terminal kinase and caspase proteases (25). Cytotoxic effects of MG are considered to be the result of DNA damage because MG is capable of inducing DNA modification and DNA-protein cross-link (29). On the other hand, our results indicated that endogenous MG prevents cells from undergoing apoptosis by maintaining Hsp27 anti-apoptotic activity.
apoptotic effects may depend on the expression levels of MG-modified protein, including Hsp27 (Fig. 5, D and E).

In conclusion, we found that MG can modify Hsp27 in cells and that this modification contributes to oligomerization and protection from caspase-dependent cell death. Hsp27 expression is involved in tumorigenicity (42) and tumor malignancy (26, 43). Also, the intracellular levels of MG should be increased in some tumor cells with a high glycolytic activity (6, 7). Thus, these studies provide information that could aid in developing cancer chemotherapeutic approaches by preventing Hsp27 anti-apoptotic activity.

Acknowledgments—We thank Drs. A. Tomida, M. Naito, and N. Fujita for helpful discussions.

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Modulation of Heat-shock Protein 27 (Hsp27) Anti-apoptotic Activity by Methylglyoxal Modification
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J. Biol. Chem. 2002, 277:45770-45775.
doi: 10.1074/jbc.M207485200 originally published online September 10, 2002

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