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GLO1 Overexpression in Human Malignant Melanoma

Warner B Bair III^a, Christopher M Cabello^a, Koji Uchida^b, Alexandra S Bause^a, and Georg T Wondrak^{a,*}

^a College of Pharmacy & Arizona Cancer Center, Department of Pharmacology and Toxicology, University of Arizona, Tucson, AZ 85724, USA

^b Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8601, Japan

Abstract

Glyoxalase I [lactoylglutathione lyase (EC 4.4.1.5) encoded by GLO1] is a ubiquitous cellular defense enzyme involved in the detoxification of methylglyoxal, a cytotoxic byproduct of glycolysis. Accumulative evidence suggests an important role of GLO1 expression in protection against methylglyoxal-dependent protein adduction and cellular damage associated with diabetes, cancer, and chronological aging. Based on the hypothesis that GLO1 upregulation may play a functional role in glycolytic adaptations of cancer cells, we examined GLO1 expression status in human melanoma tissue. Quantitative RT-PCR analysis of a cDNA tissue array containing 40 human melanoma tissues (stages III and IV) and 13 healthy controls revealed pronounced upregulation of GLO1 expression at the mRNA level. Immunohistochemical analysis of a melanoma tissue microarray confirmed upregulation of glyoxalase 1 protein levels in malignant melanoma tissue versus healthy human skin. Consistent with an essential role of GLO1 in melanoma cell defense against methylglyoxal cytotoxicity, siRNA interference targeting GLO1-expression (siGLO1) sensitized A375 and G361 human metastatic melanoma cells towards the antiproliferative, apoptogenic, and oxidative stress-inducing activity of exogenous methylglyoxal. Protein adduction by methylglyoxal was increased in siGLO1-transfected cells as revealed by immunodetection using a monoclonal antibody directed against the major methylglyoxal-derived epitope argpyrimidine that detected a single band of methylglyoxal-adducted protein in human LOX, G361, and A375 total cell lysates. Using 2D-proteomics followed by mass spectrometry the methylglyoxal-adducted protein was identified as heat shock protein 27 (Hsp27; HSPB1). Taken together, our data suggest a function of GLO1 in the regulation of detoxification and target-adduction by the glycolytic byproduct methylglyoxal in malignant melanoma.

Keywords

Malignant melanoma; glyoxalase 1; methylglyoxal; heat shock protein 27; protein adduction

Introduction

Glyoxalase 1 [lactoylglutathione lyase (EC 4.4.1.5), encoded by GLO1 (GeneID: 2739)] is a ubiquitous cellular defense enzyme involved in the detoxification of methylglyoxal, a cytotoxic byproduct of glycolysis [1–3]. The glyoxalase system comprising glyoxalase I, glyoxalase II, and reduced glutathione transforms electrophilic reactive α -oxoaldehydes including

* Address correspondence to: Georg T. Wondrak, Ph.D., University of Arizona, Arizona Cancer Center, 1515 North Campbell Avenue, Tucson, AZ 85724 USA, wondrak@pharmacy.arizona.edu, Telephone: 520-626-9017, FAX: 520-626-3797.

Competing interests

The authors declare that they have no competing interests.

methylglyoxal into the corresponding non-cytotoxic α -hydroxyacids. First, glyoxalase I catalyzes the isomerization of the hemithioacetal-adduct formed nonenzymatically between methylglyoxal and glutathione into the corresponding thioester (S-2-hydroxypropionylglutathione). Glyoxalase II then catalyses thioester hydrolysis with formation of D-lactate and free glutathione.

Accumulative evidence suggests an important role of GLO1 expression in the suppression of methylglyoxal-dependent protein adduction and cellular damage associated with diabetes, cancer, and chronological aging [1,3–5]. For example, overexpression of GLO1 has been shown to limit formation of methylglyoxal-derived protein epitopes called advanced glycation endproducts (AGEs) in endothelial cells suggesting a protective role of GLO1-expression in diabetic microangiopathy [6]. Intracellular methylglyoxal formation is increased under conditions of high glycolytic flux such as hyperglycemia associated with diabetes [6] and aerobic glycolysis associated with malignant transformation and tumor progression [3,7–9]. Indeed, many cancer cell lines are known to overexpress GLO1, which may reflect a cellular response to elevated cellular methylglyoxal stress associated with glycolytic adaptations of cancer cells, commonly referred to as the ‘Warburg’ effect [9–11]. Overexpression of GLO1 has been documented in numerous cancer cell lines and human tumor tissues including invasive ovarian cancer, prostate carcinoma, and breast cancer [3,10,12–14]. Moreover, GLO1 overexpression has been associated with cancer cell survival and resistance to chemotherapeutic agents [3,15,16]. Recent research indicates that glyoxalase 1 may be a valid molecular target for cancer chemotherapy, and pharmacological inhibitors of glyoxalase 1 have shown anticancer activity *in vitro* and *in vivo* [10,17,18].

Melanoma is a malignant tumor of melanocytes that causes the majority of skin cancer-related deaths [19,20]. Glycolytic flux in metastatic melanoma cells is elevated as indicated by expression and secretion of lactate dehydrogenase, an important prognostic marker in the metastatic phase of the disease thought to be related to the hypoxic environment of tumour metastases [11,21]. It has been demonstrated earlier that overexpression of Akt converts radial growth melanoma to vertical growth melanoma and impairs the bioenergetic function of mitochondria at the level of complex I activity with concurrent activation of glycolytic metabolism [22]. Other research has suggested the involvement of glycation-damage in melanoma cell proliferative control and metastasis, but only little information is available on expression and function of GLO1 in melanoma [20,23–25].

Here, we report for the first time that (I) GLO1 gene expression is upregulated at the mRNA and protein level in human metastatic melanoma tissue, that (II) genetic antagonism of GLO1 expression sensitizes human metastatic melanoma cells to methylglyoxal-induced cytotoxicity, and that (III) heat shock protein 27 is a target of posttranslational modification by methylglyoxal modulated by GLO1-expression in metastatic melanoma cells.

Materials and methods

Chemicals

All chemicals were from Sigma Chemical Co, St. Louis, MO.

Cell culture

G-361 human melanoma cells from ATCC (Manassas, VA, USA) were cultured in McCoy’s 5a medium containing 10% BCS. A375 and LOX melanoma cells from ATCC were cultured in RPMI medium containing 10% BCS and 2 mM L-glutamine. Primary human epidermal melanocytes (adult skin, lightly pigmented: HEMa-LP from Cascade Biologics, abbreviated HEM) were cultured using Medium 154 medium supplemented with HMGS-2 growth

supplement. HEM cells were passaged using recombinant trypsin/EDTA and defined trypsin inhibitor. Cells were maintained at 37 °C in 5% CO₂, 95% air in a humidified incubator.

GLO1 expression analysis using melanoma tissue qPCR array technology

Using a human melanoma tissue array containing first strand cDNAs (Human Melanoma TissueScan Real-Time Panel I, MERT101, ORIGENE, Rockville, MD) prepared from 40 human melanoma tissues (stages III and IV) and 13 healthy control skin samples (HMRT102, Origene), mRNA tissue levels (GLO1 versus ACTB) were determined by quantitative RT-PCR analysis following the manufacturer's instructions using an Applied Biosystems 7000 SDS and Applied Biosystems' Assays On Demand primers specific to GLO1 (assay ID Hs00198702_m1) and ACTB (β-actin, assay ID Hs99999903_m1). Duplicate plates were analyzed for GLO1 and ACTB expression, respectively. Every tissue sample was analyzed in duplicate.

GLO1 expression analysis using tissue microarray (TMA) histochemistry

A commercial melanoma TMA (IMH-369, IMGENEX HISTO-Array™, Imgenex, San Diego, CA) containing 59 human malignant melanoma tissue specimens was analyzed by immunohistochemical staining for glyoxalase 1 epitopes. For comparison, immunohistochemical analysis of 7 healthy human skin samples on a nonmelanoma skin cancer tissue array (IMH-323, Imgenex) was performed. Immunohistochemistry was performed using the Discovery XT automated staining platform (VMSI, Ventana Medical Systems, Tucson, AZ). Deparaffinization and antigen retrieval of cells and tissue were performed online. All steps were performed on this instrument using VMSI validated reagents, including deparaffinization, cell conditioning (antigen retrieval with a borate-EDTA buffer), primary antibody staining [monoclonal mouse anti-GLO1 antibody (4C12), Novus Biologicals, Littleton, CO; dilution: 1:25], detection and amplification using a biotinylated-streptavidin-HRP and DAB (3, 3'-diaminobenzidine tetrahydrochloride) system, and hematoxylin counterstaining. Images were captured using an Olympus BX50 and Spot (Model 2.3.0) camera. Images were standardized for light intensity. TMAs were scored manually using a 20 × objective. Intensity and prevalence of staining were corroborated by a certified pathologist, and average histologic scores (H-score) were calculated as previously reported [26]. 15 samples were excluded from quantitative analysis (melanin interference with immunostaining, missing tissue spot, non-melanoma tissue specimen). Based on the percentage of cells staining with 3+ (strong), 2+ (moderate), 1+ (weak), and 0 (absent) intensity, an H-score (range 0–3) was calculated by summing the percentages of cells staining at each intensity multiplied by the weighted intensity of staining: $H\text{-score} = (\% \text{ weakly stained cells} \times 1) + (\% \text{ moderately stained cells} \times 2) + (\% \text{ strongly stained cells} \times 3)$.

Glyoxalase 1 immunoblot analysis

Melanoma cells were lysed with RIPA buffer (100 μl, 50mM Tris-HCl, pH 7.4, 150mM NaCl, 1mM EDTA, 25% deoxycholic acid, and 1% NP-40). After sample separation (30 μg protein) by SDS-PAGE (4–15% gradient gel, Bio-Rad, Hercules, CA, USA), semidry transfer onto a nitrocellulose membrane (Optitrans, Whatman, Bedford, MA, USA) was performed, followed by incubation in blocking buffer [PBST (0.1% Tween 20), 5% nonfat dry milk] for 1 hour at 25°C. Membranes were washed three times with PBST and incubated overnight at 4°C with a polyclonal mouse anti-GLO1 antibody diluted 1:1000 (Novus Biologicals, Littleton, CO) in incubation buffer (PBST, 5% BSA). Membranes were washed three times with PBST followed by incubation for 1 hour at 25°C with HRP-linked anti-mouse IgG antibody (Cell Signaling) diluted 1:2000 in blocking buffer. Visualization occurred by enhanced chemiluminescence.

siRNA GLO1 transfection

G361 and A375 cells were transiently transfected with a 100 nMol pool of four siRNA oligonucleotides targeting GLO1 or a 100 nMol pool of four non-targeting siRNA oligonucleotides using the DharmaFECT 1 transfection reagent (Dharmacon RNA Technologies, Lafayette, CO, USA) following a standard procedure [27]. The sequences of siGENOME GLO1 SMARTpool (GLO1 siRNA) [GenBank: NM 006708] were GAUGGCUACUGGAUUGAAA; GAGUGAAGGAUCCUAAGAA; CUUCUUGGCUUAUGAGGAU; and CUUCUUGGAAUGACGCUAA. The oligos were resuspended in the Dharmacon 1× siRNA buffer and incubated in serum free media for 5 min. DharmaFECT 1 was also incubated in serum free media for 5 min prior to the addition of the siRNA oligos. The oligos were incubated with the transfection reagent for 20 minutes prior to cellular treatment. Complete media was added to the siRNA oligo mixture and the cells were incubated with the siRNAs in appropriate cell culture conditions for 72 h. Cells were then re-transfected with another 100 nMol pool of four siRNA oligonucleotides targeting GLO1 or a 100 nMol pool of four non-targeting siRNA oligonucleotides. Twenty-four hours after the second transfection, cells were either harvested for analysis.

GLO1 expression analysis by real time RT-PCR

For GLO1 expression analysis by real time RT-PCR, total cellular RNA (3×10^6 cells) was prepared using the RNEasy kit from Qiagen (Valencia, CA, USA). Reverse transcription was performed using TaqMan Reverse Transcription Reagents (Roche Molecular Systems, Branchburg, NJ, USA) and 200 ng of total RNA in a 50 μ l reaction. Reverse transcription was primed with random hexamers and incubated at 25 °C for 10 min followed by 48 °C for 30 min, 95 °C for 5 min, and a chill at 4 °C. Each PCR reaction consisted of 3.75 μ l of cDNA added to 12.5 μ l of TaqMan Universal PCR Master Mix (Roche Molecular Systems), 1.25 μ l of gene-specific primer/probe mix (Assays-by-Design; Applied Biosystems, Foster City, CA) and 7.5 μ l of PCR water. PCR conditions were: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, alternating with 60 °C for 1 min using an Applied Biosystems 7000 SDS and Applied Biosystems' Assays On Demand primers specific to GLO1 (assay ID Hs00198702_m1) and GAPDH (assay ID Hs99999905_m1). Gene-specific product was normalized to GAPDH and quantified using the comparative ($\Delta\Delta C_t$) Ct method as described in the ABI Prism 7000 sequence detection system user guide [28]. Expression values were averaged across three independent experiments, and standard deviation was calculated for graphing.

Glyoxalase 1 specific enzymatic activity

Glyoxalase I specific enzymatic activity in melanoma cell cytosolic fractions was analyzed following a published standard procedure [6]. Briefly, cells were harvested by trypsinization and lysed in PBS with protease inhibitors by three cycles of freezing and thawing with sonication. After centrifugation (12,000 g, 10 min, 4 °C), the supernatant cytosolic fraction was analyzed for protein content (BCA assay, Pierce) and then examined for enzymatic activity by spectrophotometric analysis. After equilibration of the standard assay mixture (8 mM MG, 1 mM reduced glutathione, 16 mM MgSO₄, 200 mM imidazole, pH 7) for hemithioketal formation (2 min, 25 °C), the reaction was initiated by addition of the cytosolic extract (10 μ g protein) monitoring the increase in absorbance at 240 nm ($\epsilon = 3.37 \text{ mM}^{-1} \text{ cm}^{-1}$) indicating formation of S-D-lactoylglutathione for 2 min at 25 °C. One unit of activity is defined as the formation of 1 μ mol of S-D-lactoylglutathione/min/mg cell protein.

Cell proliferation assay

A published standard procedure was followed [28]. Cells were seeded at 10,000 cells/dish on 35-mm dishes. After 24 h, cells were treated with test compound. Cell number at the time of

compound addition and 72 h later were determined using a Z2 Analyzer (Beckman Coulter, Inc., Fullerton, CA, USA). Proliferation was compared with cells that received mock treatment. The same methodology was used to establish IC₅₀ values (methylglyoxal concentration that induces 50% inhibition of proliferation of treated cells within 72 h exposure $\pm$ SD, n = 3) of anti-proliferative potency.

Apoptosis analysis

Induction of cell death was confirmed by annexin-V-FITC/propidium iodide (PI) dual staining of cells followed by flow cytometric analysis as published earlier [29]. Cells (100,000) were seeded on 35 mm dishes and received treatment 24 hours later. For apoptosis analysis, cells were harvested by trypsinization and cell staining was performed using an apoptosis detection kit according to the manufacturer's specifications (APO-AF, Sigma, St. Louis, MO).

Detection of intracellular oxidative stress by flow cytometric analysis

Induction of intracellular oxidative stress by methylglyoxal exposure was analyzed by flow cytometry using 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) as a sensitive non-fluorescent precursor dye according to a published standard procedure [30]. Human A375 melanoma cells were treated with methylglyoxal (500 μ M, 4 h) followed by DCFH-DA loading. To this end, cells were incubated for 60 min in the dark (37°C, 5% CO₂) with culture medium containing DCFH-DA (5 μ g/mL final concentration). Cells were then harvested, washed with PBS, resuspended in 300 μ l PBS and immediately analyzed by flow cytometry.

Proteomic identification of MG-adducted proteins in human melanoma cells

2D-gel electrophoresis was performed following standard procedures established at the SWEHS-Proteomics Core Facility available online (www.probase.org). 1×10^6 cells were directly extracted using the isoelectric focusing sample buffer and 140 μ g total cellular protein was loaded onto a 12.5% gel for two-dimensional electrophoresis (pH range 5–8). After the run, proteins were visualized by silver staining and gel images are captured using the Investigator ProPic imager (Genomic Solutions). A duplicate gel run under the same conditions underwent protein transfer onto a nitrocellulose membrane using a semidry transfer system. MG-adducted proteins were visualized using a commercial murine primary monoclonal antibody (mAb3C, provided by Koji Uchida, Nagoya University, Japan or obtained commercially from NOF Corporation, Japan; dilution 1:10,000) directed against the methylglyoxal-adduction product arg-pyrimidine as described below [41]. Immunoreactive MG-adducted proteins from parallel silver stained gels were then subjected to mass spectrometric identification. Automated spot picking was performed using the Genomic Solutions Investigator ProPic™ system. After in-gel trypsin digestion following a standard procedure, LC-MS-MS analysis was performed in the data dependent scanning mode using a Michrom paradigm nano-LC-system and a LCQ-Deca-XPplus tandem-MS system with nanospray interface. Protein sequences of tryptic fragments were identified using Turbo SEQUEST analysis software-based searches. The Swiss-Prot or nr databases were searched within a peptide mass tolerance of $\pm$ 1.5 Da and a fragment mass tolerance of $\pm$ 1.5 Da with two missed cleavages allowed. Alkylation of a cysteine residue and the oxidation of methionine were considered as modifications. Proteins were evaluated by considering the number of matched tryptic peptides, the percentage coverage of the entire protein sequence, the apparent M_w, and the pI of the protein.

Western blot analysis of Hsp27 and MG-adducted protein

Western blot analysis was performed on total cell protein extracts after separation by 12% SDS-PAGE (20 μ g protein in 15 μ l 1 $\times$ SDS-PAGE buffer). After transfer to nitrocellulose using a semi-dry blotting system (Biorad), membranes were blocked and then probed according to

standard procedures using the following primary antibodies (overnight at 4 °C): monoclonal mouse anti-argpyrimidine IgG (mAb3C; 1:10,000, for sources see above), polyclonal rabbit anti-human Hsp27 (Stressgen; SPA-803, 1:5000). After incubation (1:10,000, 1 hr at room temperature) with secondary HRP-conjugated goat anti-rabbit and goat anti-mouse antibodies (Jackson Laboratories), respectively, visualization was performed using enhanced chemiluminescence.

Statistical analysis

Unless indicated differently, the results are presented as means $\pm$ S.D. of at least three independent experiments. They were analyzed using the two-sided Student's *t* test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

Results

GLO1 gene expression is upregulated at the mRNA and protein level in human malignant melanoma tissue

Accumulative experimental evidence suggests that GLO1 upregulation plays a functional role in glycolytic adaptations of various cancers. We therefore examined GLO1 expression status in human melanoma at the mRNA level by quantitative RT-PCR analysis using a tissue array containing first strand cDNAs prepared from 40 human melanoma tissues (stages III and IV) and 13 healthy controls from unaffected human skin. For normalization, ACTB (β -actin) expression levels were determined. Average GLO1 mRNA expression levels in stage III tissues were increased approximately 4 fold [mean $\pm$ SEM: 4.20 ± 0.57 ; $n = 21$ (** $p < 0.001$)] over average healthy control (1.15 ± 0.17 ; $n = 13$). Average GLO1 mRNA expression levels in stage IV tissues were increased approximately 10 fold [10.36 ± 3.20 ; $n = 21$ (* $p < 0.05$)] over average healthy control tissues.

Next, GLO1 expression in melanoma was examined by immunohistochemical detection of glyoxalase 1-protein levels in a TNM-staged melanoma tissue microarray [TMA, IMH-369 (IMGEX HISTO-ArrayTM)] containing 44 human malignant melanoma tissue specimens and was compared to 7 healthy skin samples (control, IMH-323) (Fig. 2). For every tissue, average histological (H) scores were calculated by multiplying staining intensity by prevalence. Exemplary specimens are presented in Fig. 2C. H-score analysis for immunohistochemical comparison between healthy tissue and melanoma specimens revealed a significant upregulation of glyoxalase 1 protein levels in malignant melanoma tissue ($n = 44$) (Fig. 2A). Average glyoxalase 1 immunostaining in stage II/III tissues was increased approximately 7 fold [mean $\pm$ SEM: 1.38 ± 0.14 ; $n = 33$ (** $p < 0.001$)] over average healthy control (0.19 ± 0.12 ; $n = 7$) as summarized in Fig. 2B. Average glyoxalase 1 immunostaining in stage IV tissues was increased approximately 9 fold [1.80 ± 0.33 ; $n = 11$ (** $p < 0.01$)] over average healthy control tissues, but immunohistochemical differences between stage II/III versus stage IV did not reach the level of statistical significance (n.s.).

Genetic antagonism of GLO1 expression sensitizes human metastatic melanoma cells to methylglyoxal-induced cytotoxicity

Earlier work has implicated GLO1 expression as an enzymatic defense mechanism involved in the detoxification of methylglyoxal, a cytotoxic byproduct of glycolysis [1–3,16,25]. We therefore examined the hypothesis that genetic antagonism of GLO1 expression sensitizes cultured human melanoma cells to methylglyoxal-associated cytotoxicity. First, constitutive GLO1 expression was confirmed in three metastatic human melanoma cell lines (G361, A375, LOX) by immunoblot analysis (Fig. 3A). Next, efficacy of genetic antagonism of GLO1 expression by siRNA-interference (siGLO1) was confirmed at the mRNA (Fig. 3C) and protein level (Fig. 3B) in A375 and G361 metastatic melanoma cells. Massive downregulation of

GLO1 mRNA levels by 90% versus control levels was achieved by siRNA-interference (Fig. 3C). Moreover, siRNA-induced downregulation of glyoxalase 1 specific enzymatic activity by approximately 80% (0.27 ± 0.14 u) versus untreated (1.60 ± 0.09 u) and siControl-treated wildtype (1.68 ± 0.22 u) was observed in G361 melanoma cells (Fig. 4D). An equally pronounced reduction of glyoxalase 1 specific activity was achieved in siRNA treated A375 cells (data not shown). GLO1 expression status was also examined in primary human epidermal melanocytes (HEM) by immunoblot analysis (Fig. 3D, insert) and determination of specific enzymatic activity (Fig. 3D) that revealed an attenuated level of expression (approximately 65% reduction of protein levels according to densitometric immunoblot analysis; almost 50% reduction of specific enzymatic activity) as compared to G361 cells.

Next, it was demonstrated that genetic antagonism of GLO1 expression sensitizes A375 and G361 melanoma cells to the antiproliferative activity of methylglyoxal (Fig. 3E–F). In wildtype (wt) and controlsiRNA (siControl) treated A375 cells, the dose response relationship of inhibition of proliferation by methylglyoxal was almost identical [IC₅₀ (μM): (278 ± 13 , 72h continuous exposure). In contrast, siRNA interference targeting GLO1 (siGLO1) sensitized A375 cells, inducing a left shift of the dose response curve [IC₅₀ (μM): ($87.4 \mu\text{M} + 5.1$, 72h continuous exposure). Similar results were obtained in G361 melanoma cells as demonstrated in Fig. 3F where exposure to methylglyoxal (100 μM, 72 h continuous exposure) reduced proliferation in siGLO1 treated cells by almost 70% of control levels, whereas siControl treated cells displayed only a moderate inhibition of proliferation (20%) in response to methylglyoxal treatment.

At higher doses of methylglyoxal (500 μM, 24 h exposure) induction of apoptosis as assessed by flow cytometric analysis was strongly enhanced in A375 cells after siGLO1 transfection (Fig. 3G). In siControl-transfected cells, methylglyoxal treatment moderately reduced the percentage of viable cells (86.0 ± 1.3 % of total gated cells), whereas in siGLO1-transfected cells methylglyoxal treatment strongly impaired viability with only 49.2 ± 4.7 % of total gated cells surviving after 24 h exposure.

Cellular treatment with reactive α-dicarbonyl compounds including methylglyoxal has been shown earlier to induce formation of reactive oxygen species (ROS) causing cellular oxidative stress [38]. Consistent with these earlier findings siGLO1-transfection strongly sensitized A375 melanoma cells to induction of oxidative stress by methylglyoxal (500 μM, 4 h), detected by flow cytometry using using 2', 7'-dichlorodihydrofluorescein diacetate (DCFH-DA) as a sensitive non-fluorescent precursor dye (Fig. 3H). After siGLO1-transfection, methylglyoxal-treatment induced an approximately eleven-fold increase in cellular peroxide levels, whereas methylglyoxal-treatment of siControl-transfected cells resulted only in an approximately 3-fold increase in peroxide-specific dichlorofluorescein (DCF) cellular fluorescence intensity.

Proteomic identification of human Hsp27 as a target of posttranslational modification by methylglyoxal modulated by GLO1 expression in metastatic melanoma cells

After demonstrating cell sensitization to methylglyoxal-induced cytotoxicity in response to genetic GLO1 antagonism, we examined the hypothesis that methylglyoxal-adduction of protein targets may be increased in siGLO1-transfected cells. To this end, immunodetection of argpyrimidine, a major protein-AGE-epitope derived from spontaneous methylglyoxal-adduction of arginine-residues [41], was performed in total protein extracts from G361, LOX, and A375 human melanoma cells. Western analysis using a monoclonal antibody directed against argpyrimidine (mAB3C, [41]) identified a single band of methylglyoxal-adducted protein of approximately 26 kDa molecular weight detected in human LOX, G361, and A375 total lysates (Fig. 4A). Importantly, siRNA-interference targeting GLO1 but not transfection with siControl upregulated cellular levels of the methylglyoxal-adducted protein in G361 and A375 melanoma cells (Fig. 4B).

Next, Western analysis of methylglyoxal-adduction of the complete melanoma proteome was performed as summarized in Fig. 4C–D. After 2D-separation of total G361 melanoma protein extracts by isoelectric point (pH 5–8) and molecular mass (10–250 kDa; visualized by silverstaining, Fig. 4C, panel I) on duplicate gels, 2D-Westernblot analysis of argpyrimidine-modified target proteins identified two immunoreactive spots indicative of methylglyoxal-adduction (Fig. 4C, panel II). Both methylglyoxal-adducted protein spots of apparent identical molecular weight (approximately 26 kDa), but different pIs were selected from the original silver-stained 2D-gel and trypsin-digested. The trypsinized spots, analyzed by LC-MS-MS and Sequest/nr fasta data base search, revealed the identity of both proteins as human heat shock protein 27 (Hsp27). For spot 1 (pI 6.7), four tryptic peptides were sequenced and matched to the complete sequence (residues 28–37, 57–75, 97–112, and 172–188; 30.2% coverage by amino acid count) of human heat shock protein 27kDa protein 1 (HSPB1; *NCBI: NP_001531.1*; *REFSEQ: NM_001540.2*) with high cross correlation values as summarized in Fig. 4D. For the less abundant, more acidic spot 2 (pI 6.1), one tryptic peptide (residues 97–112; 7.8% coverage by amino acid count) was matched to the same protein (data not shown).

Since phosphorylation and methylglyoxal-adduction both result in protein acidification by introduction of a negative charge or removal of a positive charge, respectively, the appearance of a second, more acidic variant observed at lower pI is consistent with a different posttranslational phosphorylation and/or methylglyoxal-adduction status. Indeed, probing the stripped membrane with a polyclonal antibody directed against total Hsp27 insensitive to posttranslational adduction status suggests that a significant proportion of the total Hsp27 pool in G361 human melanoma cells is methylglyoxal-adducted (Fig. 4C, panel III; two center arrows). However, at least two Hsp27 subtypes with no detectable methylglyoxal-adduction are present (Fig. 4C, panel III; right and left arrows) suggesting an unresolved complexity of Hsp27 posttranslational modification and regulation in melanoma cells.

Discussion

It is widely accepted that glycolytic alterations of cancer cell energy metabolism represent a metabolic adaptation to tumor hypoxia transcriptionally controlled by hypoxia inducible factor (HIF-1 α) [31,32]. In addition, recent studies suggest additional functions of glycolytic enzymes and metabolites including methylglyoxal as signaling molecules involved in the regulation of tumor cell survival [9,20,24,33–36].

In this study tissue microarray technology revealed dramatic overexpression of the methylglyoxal-defense enzyme glyoxalase I in malignant human melanoma tissue, both at the mRNA and protein levels (Figs. 1–2). Consistent with an essential role of GLO1 expression in cellular defense against methylglyoxal cytotoxicity, siRNA interference targeting GLO1 sensitized A375 and G361 human melanoma cells towards the well established antiproliferative, apoptogenic, and oxidative stress-inducing activity of exogenous methylglyoxal (Fig. 3) [3,15,37–39]. Remarkably, proliferative capacity and viability remained unchanged in siGLO1transfected cells that were unchallenged by exposure to exogenous methylglyoxal (Fig. 3E–G). This suggests that under standard normoxic cell culture conditions employed in our experiments GLO1-downregulation is tolerated without compromising proliferative capacity of A375 and G361 melanoma cells. Future studies will therefore examine the effects of pharmacological or genetic antagonism of GLO1-expression on melanoma cell viability and proliferation under hypoxic conditions, characteristic of the tumor microenvironment known to be associated with increased glycolytic flux that may potentiate melanoma cell dependence on glyoxalase function.

The glycolytic byproduct methylglyoxal is a reactive electrophilic metabolite that modifies cysteine-, lysine-, and arginine-residues of specific target proteins by covalent adduction

referred to as glycation [3]. Glycation occurs with formation of crosslinks and heterocyclic advanced glycation endproduct (AGE)-epitopes such as the arginine-derivatives N^δ-(5-methylimidazolin-4-one-2-yl)-ornithine (MG-H1) [40] and argpyrimidine [41]. Apart from exerting general cytotoxic effects associated with protein crosslinking and mutagenic adduction of DNA bases, methylglyoxal-induced posttranslational adduction of selected target proteins such as the transcriptional repressor mSin3A [42], mitochondrial permeability transition pore constituents [35,43], and heat shock proteins [9,34,44] is rapidly emerging as a novel mechanism of transcriptional control and cancer cell survival signaling. Remarkably, reversible adduction of arginine residues contained in mitochondrial permeability transition pore constituents does not occur with formation of stable protein epitopes amenable to immunodetection suggesting functional consequences of methylglyoxal-adduction beyond the formation of stable adducts [35].

In our experiments employing genetic antagonism of GLO1 expression in G361 and A375 melanoma cells, we noticed upregulation of cellular levels of a methylglyoxal-adducted protein that was then identified using proteomic methodologies as Hsp27 (Fig. 4). Hsp27 overexpression is observed in a wide range of human tumors and cancer cell lines and overexpression is associated with poor prognosis and resistance to chemotherapy [45–48]. In melanoma, overexpression of Hsp27 has been correlated with metastatic progression, and a recent unbiased proteomic screen for proteins overexpressed in melanoma identified Hsp27 and Hsp60 among a total of six proteins [49,50]. Hsp27 modulation of cell survival has been attributed to four types of molecular variables: total expression levels, oligomerization status, phosphorylation pattern, and methylglyoxal-adduction [44,45,51]. Hsp27 phosphorylation by the p38 MAP kinase/MAPKAP kinase 2-cascade occurs at three different sites (Ser15, Ser78, Ser82). Hsp27 phosphorylation induces disaggregation with formation of small Hsp27 oligomers (<100 kDa) and attenuated Hsp27 chaperone function [44]. In contrast, methylglyoxal-adduction induces Hsp27 aggregation leading to inhibition of apoptosome assembly, cytochrome C inactivation, inhibition of caspase 3, and suppression of apoptosis [8,34,44,52]. In glomerular mesangial cells, methylglyoxal-adduction of Hsp27 has been shown to impair Hsp27 antiapoptotic activity by decreasing its binding to cytochrome C [53]. The occurrence of methylglyoxal-adducted Hsp27 has been documented in various non-melanoma cancer cell lines (NCI-H23 lung cancer, U937 leukemia, and PC3 prostate cancer) [34] and has recently been observed in human non-small cell lung cancer tissue associated with increased chemoresistance to cisplatin treatment [8,9]. Mechanistic studies involving site-directed mutagenesis have established that Arg-188 is the site of methylglyoxal adduction of Hsp27 observed in 293T cells, a modification that is essential to methylglyoxal-dependent Hsp27 repression of cytochrome c-mediated caspase activation [34].

Accumulative experimental evidence suggests a complex role of cellular carbonyl stress mediated through protein adduction by the glycolytic byproduct methylglyoxal in the regulation of cell proliferation, viability, and inflammatory signaling. On one side, methylglyoxal exerts antiproliferative and apoptogenic effects that include protein-crosslinking, formation of reactive oxygen species (ROS) with apoptosis signaling kinase 1 (ASK1)-activation, and genotoxicity [3,38,39]. On the other side, methylglyoxal-adduction of selected cellular protein targets including components of the mitochondrial permeability transition pore complex, Hsp27, and the transcriptional repressor mSin3A may serve specific cellular functions in support of cell viability and increased inflammatory signaling [9,34,35, 42–44]. In analogy to these opposing cellular effects of methylglyoxal, a double-edged role of cytotoxic ROS acting as mitogenic and anti-apoptotic signaling molecules involved in redox control of melanoma cell proliferation and viability has been substantiated recently [22,36, 54–58].

Our data demonstrate for the first time pronounced upregulation of GLO1 expression in human malignant melanoma tissue. Furthermore, our data document the increased vulnerability of siGLO1-transfected melanoma cells to the cytotoxic effects of exogenous methylglyoxal. Remarkably, siGLO1-transfection of melanoma cells induces upregulation of cellular levels of methylglyoxal-adducted Hsp27, an emerging antiapoptotic factor observed in various cancer cell lines and tissues [9,34,44]. Taken together, these data suggest a bimodal function of glyoxalase 1 in the regulation of cellular carbonyl stress originating from methylglyoxal-adduction. Acting as a crucial regulator of methylglyoxal levels and target-adduction in melanoma cells, GLO1 expression may balance and determine the opposing effects of methylglyoxal cytotoxicity and cytoprotection, a hypothesis to be substantiated by future experiments.

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Figure 1. GLO1 mRNA levels are upregulated in human malignant melanoma tissue

(A) Using a human melanoma tissue-scan real-time panel containing first strand cDNAs prepared from 40 human melanoma tissues (stages III and IV) and 13 healthy controls, mRNA tissue levels (GLO1 versus ACTB) were determined by quantitative RT-PCR analysis. Every tissue sample was analyzed in duplicate. (B) Dot blot depiction of GLO1 mRNA expression levels. Comparison between groups [control (n=13); stage III (n=21); stage IV (n=19)] was done using the two-sided Student's t-test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; lines indicate average values).

Figure 2. Immunohistochemical detection of glyoxalase 1-epitopes in human malignant melanoma tissue

(A) 44 Human malignant melanoma tissue specimens on a TNM-staged melanoma TMA (IMH-369, IMGENEX HISTO-Array™, depicted in panel C, left portion) containing 44 human malignant melanoma tissue specimens and 7 healthy control skin samples (control, IMH-323) were analyzed by immunohistochemical staining for glyoxalase 1. Average histological (H) scores were calculated by multiplying intensity by prevalence. (B) H-score analysis depicted as dot blot. Comparison between groups [control (n=7); stage II-III (n=33); stage IV (n=11)] was done using the two-sided Student's t-test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; lines indicate average values). (C) Melanoma-TMA IMH-369 (left) with tissue samples that represent the range of H-scores: (from left to right) non-melanoma healthy control (H-score: 0), melanoma samples (H-scores: I-III).

Figure 3. Genetic antagonism of GLO1 expression by RNA-interference sensitizes human metastatic melanoma cells to methylglyoxal-induced inhibition of proliferation, apoptosis, and oxidative stress

(A) Immunoblot analysis of GLO1 expression in three metastatic human melanoma cell lines. (B–C) siRNA-interference (siGLO1) downregulates GLO1 mRNA (C) and glyoxalase 1 protein levels (B) in A375 and G361 metastatic melanoma cells. (siCtr: non-targeting siRNA oligonucleotides). (D) siRNA-interference downregulates glyoxalase 1 specific enzymatic activity in G361 melanoma cells (wt: non-transfected wildtype cells). The insert compares glyoxalase 1 protein levels in G361 metastatic melanoma cells and primary human epidermal melanocytes (HEMs) by immunoblot analysis. (E) Genetic antagonism of GLO1 expression sensitizes A375 melanoma cells to the antiproliferative activity of methylglyoxal determined as specified in Materials and Methods. (F) Genetic downregulation of GLO1 expression sensitizes G361 melanoma cells to the antiproliferative activity of methylglyoxal (100 μ M, 72 h). (G) Genetic antagonism of GLO1 expression sensitizes A375 melanoma cells to the apoptogenic activity of methylglyoxal (500 μ M, 24 h) as assessed by flow cytometry. The numbers indicate viable cells (AV⁺, PI⁺, lower left quadrant) in percent of total gated cells (mean $\pm$ SD, n=3). (H) Genetic antagonism of GLO1 expression sensitizes A375 melanoma cells to induction of oxidative stress by methylglyoxal (500 μ M, 4 h) determined by flow cytometry as specified in Materials and Methods. Data points are depicted as means $\pm$ SD (n $\geq$ 3) and statistical comparison between individual data points was performed using the two-sided Student's *t* test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

Figure 4. Proteomic identification of human Hsp27 as a target of posttranslational modification by methylglyoxal modulated by GLO1-expression in human metastatic melanoma cells

(A) Detection of a methylglyoxal-adducted protein in total melanoma cell (G361, A375, LOX) lysates as assessed by 12% SDS-PAGE followed by Western transfer and immunodetection using the monoclonal antibody mAB3C directed against the methylglyoxal-specific adduct argpyrimidine as specified in Materials and Methods.

(B) GLO1-targeting siRNA-interference upregulates cellular levels of a methylglyoxal-adducted protein in G361 and A375 melanoma cells as assessed by immunodetection performed as in (A).

(C, panel I) 2D-gel of total G361 human melanoma cell protein extract; 1×10^6 cells were directly extracted using the isoelectric focusing sample buffer and 140 μ g total cellular protein was loaded onto a 12.5% gel for two-dimensional electrophoresis (pH range 5–8). After the run, proteins were visualized by silver staining (shown in C, panel I) and a duplicate gel run under the same conditions underwent protein transfer onto a nitrocellulose membrane.

(C, panel II) 2D-Westernblot for methylglyoxal-adducted proteins using the monoclonal antibody mAB3C. Both methylglyoxal-adducted protein spots of apparent identical molecular weight but different pI [blue (spot 1) and red (spot 2) circles] were selected from the original silver-stained 2D-gel and trypsin-digested. The trypsinized spots were then identified by LC-MS-MS analysis and Sequest/nr fasta data base search. Both methylglyoxal-adducted proteins are identical with Hsp27 as specified in (D).

(C, panel III) After stripping, the nitrocellulose membrane was reprobed for total Hsp27 using a polyclonal rabbit antibody directed against human Hsp27. At least four different Hsp27 subtypes of different pI were detected (arrows), two of which colocalized with the methylglyoxal-adducted proteins detected in (C, panel II).

(D) Sequest analysis of protein spot 1 (blue circle) picked from the gel shown in (C, panel I). Four tryptic peptides (black bars) were sequenced and matched to the complete sequence (residues 1–205, 31.2 % sequence coverage) of human Hsp27 with high cross correlation values.