Chronic depletion of glutathione exacerbates ventricular remodelling and dysfunction in the pressure-overloaded heart

Yosuke Watanabe, Kazuhiro Watanabe, Tsuyoshi Kobayashi, Yukio Saito, Daisuke Fujioka, Takamitsu Nakamura, Jun-ei Obata, Kenichi Kawabata, Hitodo Mishina, and Kiyotaka Kugiyama*

Department of Internal Medicine II, University of Yamanashi, Faculty of Medicine, 1110 Shimokato, Chuo, Yamanashi 409-3898, Japan

Received 31 May 2012; revised 20 October 2012; accepted 1 November 2012; online publish-ahead-of-print 5 November 2012

Time for primary review: 20 days

Aims Chronic depletion of myocardial glutathione (GSH) may play a role in cardiac remodelling and dysfunction. This study examined the relationship between chronic GSH depletion and cardiac failure induced by pressure overload in mice lacking the modifier subunit (GCLM) of glutamate–cysteine ligase, the rate-limiting enzyme for GSH synthesis. In addition, we examined the association between idiopathic dilated cardiomyopathy (DCM) in humans and –588C/T polymorphism of the GCLM gene, which reduces plasma levels of GSH.

Methods and results Pressure overload in mice was created by transverse aortic constriction (TAC). Myocardial GSH levels after TAC in GCLM+/− mice were 31% of those in GCLM+/+ mice. TAC resulted in greater heart and lung-weight-to-body-weight ratios, greater dilation and dysfunction of left ventricle, more extensive myocardial fibrosis, and worse survival in GCLM+/− than GCLM+/+ mice. Supplementation of GSH diethyl ester reversed the left-ventricular dilation and contractile dysfunction and the increased myocardial fibrosis after TAC in GCLM+/− mice. The prevalence of –588T polymorphism of the GCLM gene was significantly higher in DCM patients (n = 205) than in age- and sex-matched control subjects (n = 253) (36 vs. 19%, respectively, P < 0.001). The –588T polymorphism increased the risk of DCM that was independent of age, diabetes, and systolic blood pressure (OR 3.13, 95% CI: 2.28–4.44; P < 0.0001).

Conclusion Chronic depletion of GSH exacerbates remodelling and dysfunction in the pressure-overloaded heart. The clinical relevance of this mouse model is supported by a significant association between –588T polymorphism of the GCLM gene and patients with DCM.

Keywords Cardiomyopathy • Fibrosis • Gene polymorphisms • Heart failure • Oxygen radicals

1. Introduction

Left-ventricular (LV) pressure overload induces hypertrophy, fibrosis, and contractile dysfunction.1–3 When LV pressure overload is prolonged, ventricular hypertrophy can progress to dilatation and failure.1,2 It has been suggested that reduced glutathione (GSH) plays an important role in the pathogenesis of myocardial remodelling and dysfunction.4–6 A previous report demonstrated that deletion of GSH peroxidases (GPx)-1 enhanced cardiac hypertrophy in mice with angiotensin II-induced hypertension,5 suggesting a role for GSH in protecting the heart against cardiac remodelling and failure. In contrast, a recent study showed that an increase in GSH levels was associated with cardiac dysfunction and heart failure in transgenic mice with cardiac-specific overexpression of the mutant of human αB-crystallin (hR120GcryAB).6 Thus, the role of GSH in cardiac structural remodelling and dysfunction is not clear.

GSH is synthesized through the action of glutamate–cysteine ligase (GCL) and GSH synthetase.7–10 GCL is a rate-limiting enzyme for de novo GSH synthesis.7–10 GCL is a heterodimer composed of catalytic (GCLC) and modifier (GCLM) subunits.7–10 GCLC has all of the catalytic activity of GCL, whereas GCLM increases the catalytic efficiency of GCLC, although it does not have catalytic activity.7–10 Oxidative stress...
upregulates GCL genes to increase GSH synthesis, and the upregulation of these genes may provide a protective mechanism against further oxidative stress.\textsuperscript{7–10} We have previously reported that patients with T allele in polymorphism (−588C/T) in the 5′-flanking region of the GCLM gene showed a decrease in oxidant-induced GCLM gene expression (by 50% of those with the CC genotype) in monocytes cultured from peripheral blood. In addition, we found these patients had low plasma GSH levels (by 40% of the CC genotype).\textsuperscript{11,12} Furthermore, we have also shown that GCLM gene knock-out mice (GCLM\textsuperscript{−/−}) have long-term depletion of myocardial GSH levels that are only 34% of the levels in wild-type (GCLM\textsuperscript{+/+}) mice.\textsuperscript{13} Thus, the goal of this study was to examine the relationship between chronic GSH depletion and cardiac remodelling and dysfunction using GCLM\textsuperscript{−/−} mice with chronic pressure overload. To determine the clinical relevance of this experiment in mice, we also examined whether polymorphism of the GCLM gene may be associated with idiopathic dilated cardiomyopathy (DCM) in humans.

2. Methods

For details, see Supplementary material online.

2.1 Materials

Cell-culture reagents were obtained from Sigma-Aldrich (St Louis, MO, USA) and Invitrogen (Carlsbad, CA, USA).

2.2 Mice

The experimental protocol was approved by the University of Yamanashi Animal Care and Use Committee (approval reference no. 19–35), and procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, 8th Edition, 2011). GCLM\textsuperscript{−/−} (systemic deficiency in GCLM) male mice\textsuperscript{13} with a C57BL/6J background from out mice (GCLM Imaging, Andover, MA, USA) were used in the present study. The littermates of the GCLM\textsuperscript{−/−} mice were treated with an intraperitoneal injection of 7.5 mmol/kg of GSH-diethyl ester (EE) or an equivalent volume of PBS as a placebo once daily for 4 weeks after TAC. This dose of GSH-EE was selected according to our previous study.\textsuperscript{13} Echocardiography and haemodynamic measurements were performed at the end of treatment under anaesthesia as aforementioned. At the end of the study, the mice were anesthetized with 5% isoflurane and hearts were harvested for histological study and assay of SERCA2a glutathionylation and Ca\textsuperscript{2+} uptake. In another set of experiments, GCLM\textsuperscript{−/−} and GCLM\textsuperscript{+/+} mice were treated with an intraperitoneal injection of 7.5 mmol/kg of GSH-EE or PBS once daily for the last week of the 4 weeks of TAC. The heart was harvested under anaesthesia with 5% isoflurane 8 h after the injection of GSH-EE or PBS on the seventh day of the treatment to determine myocardial levels of GSH and oxidized GSH (GSSG).

2.3 Transverse aortic constriction (TAC)

Mice were anesthetized with ketamine (100 mg/kg) and xylazine (5 mg/kg) by intraperitoneal injection. After the chest cavity was exposed by cutting open the proximal portion of the sternum, aortic constriction was produced by ligation of the transverse thoracic aorta between the innominate artery and left common carotid artery. Buprenorphine (0.05 mg/kg, Otsuka Pharmaceutical, Tokyo, Japan) was given subcutaneously every 12 h after surgery for 2 days. Sham-operated mice without constriction served as controls.

2.4 Echocardiography

Mice were lightly anesthetized with ketamine (20 mg/kg) and xylazine (1 mg/kg), and two-dimensional and M-mode transthoracic echocardiography were performed according to the method described in our previous report.\textsuperscript{13} All measurements were performed using a 15 MHz phased-array probe connected to a Sonos 5500 echocardiograph (Philips Medical Imaging, Andover, MA, USA).

2.5 Haemodynamic study

Mice were anesthetized with urethane (1.0–1.2 g/kg, IP). The right carotid artery was cannulated with a microtip pressure transducer (SPR-671, Millar, Houston, TX, USA). After the measurement of aortic pressure, the catheter was advanced into the LV for the measurement of LV pressures. After the haemodynamic study, mice were placed in an anaesthesia chamber with 5% isoflurane until they were not responsive to toe pinch. Then, the heart was rapidly removed for histological study.

2.6 Preparation of heart tissue samples

At the indicated timepoints after sham operation or TAC, mice were anesthetized with 5% isoflurane in an anaesthesia chamber until they were not responsive to toe pinch, and the heart and lung were harvested for biochemical assays or weight measurements. In some experiments, the heart was stopped during diastole by injecting a 15% potassium chloride solution into the left ventricle after the mice were anaesthetized. Then, mice were transcardially perfused with phosphate-buffered saline (PBS) followed by 4% formaldehyde in PBS under pressure (100 mm Hg), and hearts were harvested for histological analysis.

2.7 GSH-diethyl ester supplementation in TAC experiments

GCLM\textsuperscript{−/−} mice were treated with an intraperitoneal injection of 7.5 mmol/kg of GSH-diethyl ester (EE) or an equivalent volume of PBS as a placebo once daily for 4 weeks after TAC. This dose of GSH-EE was selected according to our previous study. Echocardiography and haemodynamic measurements were performed at the end of treatment under anaesthesia as aforementioned. At the end of the study, the mice were anesthetized with 5% isoflurane and hearts were harvested for histological study and assay of SERCA2a glutathionylation and Ca\textsuperscript{2+} uptake. In another set of experiments, GCLM\textsuperscript{−/−} and GCLM\textsuperscript{+/+} mice were treated with an intraperitoneal injection of 7.5 mmol/kg of GSH-EE or PBS once daily for the last week of the 4 weeks of TAC.

2.8 Measurement of GSH and GSSG levels and enzymatic activities

GSH, GSSG, and γ-glutamylcysteine (γ-GC) levels were measured by high performance liquid chromatography with electrochemical detection (Model S600 CoulArray electrochemical detector, ESA, Chelmsford, MA, USA) (see Supplementary material online).\textsuperscript{14} The enzymatic activities of GCL, glutathione reductase, Mn superoxide dismutase (MnSOD), and catalase were measured as described in Supplementary material online.

2.9 Detection of oxygen free radicals in the heart and in cultured mouse cardiac fibroblasts

The concentration of oxygen free radicals in myocardial tissue was assessed using the spin trap 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl (hydroxy-TEMPO).\textsuperscript{15} Myocardial tissue was homogenized in sodium phosphate buffer and reacted with hydroxy-TEMPO. Then, the rate of ESR signal decay of hydroxy-TEMPO was calculated.

The production of intracellular oxygen free radicals in cultured mouse cardiac fibroblasts was measured by hydroxyphenyl fluorescence (HPF) (SEKISUI MEDICAL, Tokyo, Japan).

2.10 SERCA-specific Ca\textsuperscript{2+} uptake assay in myocardial homogenates

Ca\textsuperscript{2+} uptake was determined using the Millipore filtration technique.\textsuperscript{46} Ca\textsuperscript{2+} uptake in the supernatant from myocardial homogenates was initiated by addition of 5 mmol/L ATP and terminated at 2 min by filtration. To evaluate sarcoplasmic (endoplasmic) reticulum Ca\textsuperscript{2+} ATPase (SERCA)-specific Ca\textsuperscript{2+} uptake, 10 μmol/L of thapsigargin, an SERCA-specific inhibitor, was added to the Ca\textsuperscript{2+} uptake medium, and Ca\textsuperscript{2+} uptake was measured in the same sample.

2.11 Measurement of myocardial fibrosis and microvessel density

After removing the hearts, the LV was cut transversely into three pieces. The LV sections were processed, embedded in paraffin, and serially
sectioned (6 μm sections). Myocardial sections from the LV at the level of the papillary muscles were stained with Masson’s trichrome. The relative fibrotic area (% of total area) was averaged from 20 sections (at ×400 magnification). For analysis of microvessel density, myocardial sections were double stained with biontinated lectin-I (Vector Laboratory, Burlingame, CA, USA) followed by streptavidin Alexa Fluor 555 conjugates for vessels and wheat germ agglutinin Alexa Fluor 488 conjugates for myocytes (Invitrogen, Carlsbad, CA, USA). Images were obtained by a confocal microscope (Olympus FV-1000, Tokyo, Japan).

2.12 Matrix metalloproteinase-2 (MMP-2) activity and gelatin zymography

The MMP-2 activity of myocardial homogenates was assayed with the Gelatin zymography kit (Primary Cell, Sapporo, Japan). The MMP-2 activity was assessed with densitometry.

2.13 Isolation of adult mouse cardiomyocytes and cardiac fibroblasts

Hearts were excised from mice (10–12 weeks old) after anaesthesia with 5% isoflurane in an anaesthesia chamber until they were not responsive to toe pinch. Primary cultures of mouse cardiomyocytes and cardiac fibroblasts were prepared by collagenase digestion from the ventricles as described in our previous report.13

2.14 Measurements of mRNA and protein expression and SERCA2a glutathionylation

The mRNA expression levels were quantified by a two-step real-time reverse-transcription polymerase chain reaction (PCR) assay.13 The PCR primers are listed in the Supplementary material online, Table S1.

For detection of glutathionylated SERCA2a using immunoblot analysis, myocardial tissue homogenates were immunoprecipitated by a cross-link immunoprecipitation kit (Thermo Fisher Scientific, San Jose, CA, USA) using rabbit polyclonal SERCA2a antibody (#42195, Cell signaling technology, Beverly, MA, USA). Then, the samples were separated by SDS–PAGE, and the transferred membranes were stained using anti-GSH antibody (#101-A-100, VIROGEN, Watertown, MA, USA) or anti-SERCA2a antibody.

2.15 Mechanical properties and \([Ca^{2+}]_i\) transients in mouse cultured cardiomyocytes

The cells were field-stimulated at 0.5 Hz in Tyrode’s solution in the presence or absence of GSH-diethyl ester (EE) (2 mmol/L). To determine \([Ca^{2+}]_i\), transients, cardiomyocytes were incubated with 2 μmol/L Fluo 8L AM (ABD Bioquest, Sunnyvale, CA, USA) for 20 min. The cells were stimulated at 0.5 Hz in the presence or absence of GSH-EE (2 mmol/L). Fluo 8L AM was excited by light at 488 nm, and fluorescence was measured at a wavelength of 515 nm. \([Ca^{2+}]_i\) signals were shown as the F/F0 fluorescence ratio.

2.16 Human subjects

The clinical study included 205 patients with DCM. The study also included 253 consecutive control subjects with atypical chest pain. The diagnosis of DCM and the inclusion and exclusion criteria of the patients with DCM and control subjects are described in Supplementary material online (see Supplementary Methods). The clinical characteristics of patients with DCM and control subjects are also shown in Supplementary material online, Table S2. None of the DCM patients or control subjects overlapped with those used in our previous studies.11,12 All the patients and controls gave written, informed consent for the study before enrolment. The study was approved by the Ethics Committee of Yamanashi University Hospital (approval reference no. 116). The investigation conformed to the principles outlined in the 1975 Declaration of Helsinki. The sample size calculation is shown in Supplementary material online.

2.17 Genotyping

Venous blood was collected into tubes containing EDTA, and after separation of leukocytes, genomic DNA was isolated. Genotyping of the polymorphism (−588C/T) in the 5′-flanking region of the GCLM gene was performed as described in our previous report.11

2.18 Statistical analysis

Results are expressed as means ± SEM unless otherwise indicated. Mean values were compared between two groups with an unpaired t-test, and frequencies were compared with a χ² analysis or Fisher’s exact test. Comparisons among ≥3 groups were performed by one-way ANOVA with Scheffé’s F-protected procedure for post-hoc analysis. The survival rate in each group was determined by Kaplan–Meier survival analysis and rates were compared with a log-rank test. Multiple logistic regression analysis was performed in the patients to determine the risk of DCM when −588T polymorphism of the GCLM gene was present after adjustment for other confounding risk factors. A P-value < 0.05 was considered statistically significant.

3. Results

3.1 Body weight, heart weight, heart rate, and blood pressure at baseline

Myocardial GSH levels at baseline in GCLM+/− mice were 41% of those in GCLM+/+ mice. There were no significant differences in baseline heart weight, heart rate, blood pressure measured by tail-cuff plethysmography and echocardiographic data between GCLM+/− and GCLM+/+ mice (see Supplementary material online, Table S3). The body weight (BW) at baseline was slightly less in GCLM−/− than in GCLM+/+ mice (24.9 ± 0.3 vs. 25.8 ± 0.1 g at 12 weeks of age), as described previously.13

3.2 Survival rate, heart and lung weights, echocardiography, and haemodynamics after TAC

There were no operative deaths within 24 h after TAC or sham operation. Systolic blood pressure at the aortic root increased comparably in both GCLM+/+ and GCLM−/− mice 4 weeks after TAC (Table 1). The survival rate during 4 weeks of TAC was significantly lower in GCLM−/− than in GCLM+/+ mice (80 vs. 97%, respectively, n = 30 for each genotype, P < 0.01; Figure 1A). The mice that died before 4 weeks of TAC had apparent signs of heart failure (data not shown). Analysis of the surviving mice after 4 weeks of TAC showed that heart weight (HW) and lung weight (LW) were significantly increased compared with sham-operated mice in both genotypes (Table 1). The increases in the HW/BW and LW/BW ratios after TAC were significantly greater in GCLM−/− than in GCLM+/+ mice (Figure 1B and C).

Echocardiography after 4 weeks of TAC demonstrated that LV end-diastolic dimension (LVDD) and LV end-systolic dimension (LVDs) were increased more in GCLM−/− than in GCLM+/+ mice (Table 1). LV systolic function, as assessed by LV fractional shortening (LVFS) and maximum LV dp/dt, was decreased after TAC to a greater extent in GCLM−/− than in GCLM+/+ mice (Table 1). LV diastolic function, as assessed by tau, was depressed after TAC more in
**Table 1** Comparison of body, heart, and lung weights, and echocardiographic and haemodynamic parameters between the two genotypes

<table>
<thead>
<tr>
<th></th>
<th>Sham GCLM&lt;sup&gt;+/+&lt;/sup&gt;</th>
<th>GCLM&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>TAC GCLM&lt;sup&gt;+/+&lt;/sup&gt;</th>
<th>GCLM&lt;sup&gt;−/−&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>27.0 ± 0.24</td>
<td>25.2 ± 0.18&lt;sup&gt;a&lt;/sup&gt;</td>
<td>27.1 ± 0.18</td>
<td>25.3 ± 0.16&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Heart weight (mg)</td>
<td>140 ± 4.1</td>
<td>137 ± 3.2</td>
<td>248 ± 7.1&lt;sup&gt;t&lt;/sup&gt;</td>
<td>260 ± 9.3&lt;sup&gt;t&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lung weight (mg)</td>
<td>157 ± 11</td>
<td>153 ± 11</td>
<td>311 ± 16&lt;sup&gt;t&lt;/sup&gt;</td>
<td>342 ± 25&lt;sup&gt;t&lt;/sup&gt;</td>
</tr>
<tr>
<td>Echocardiography</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IVS (mm)</td>
<td>0.60 ± 0.02</td>
<td>0.63 ± 0.03</td>
<td>0.95 ± 0.06&lt;sup&gt;1&lt;/sup&gt;</td>
<td>0.95 ± 0.04&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>LVFW (mm)</td>
<td>0.63 ± 0.02</td>
<td>0.65 ± 0.02</td>
<td>0.96 ± 0.06&lt;sup&gt;1&lt;/sup&gt;</td>
<td>0.96 ± 0.04&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>LVEDd (mm)</td>
<td>3.45 ± 0.04</td>
<td>3.38 ± 0.06</td>
<td>3.66 ± 0.06&lt;sup&gt;1&lt;/sup&gt;</td>
<td>3.88 ± 0.06&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>LVSDs (mm)</td>
<td>1.85 ± 0.18</td>
<td>1.80 ± 0.08</td>
<td>2.37 ± 0.12&lt;sup&gt;1&lt;/sup&gt;</td>
<td>2.91 ± 0.07&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>LVFS (%)</td>
<td>46.5 ± 1.3</td>
<td>47.7 ± 1.1</td>
<td>35.3 ± 2.3&lt;sup&gt;t&lt;/sup&gt;</td>
<td>25.1 ± 1.5&lt;sup&gt;t&lt;/sup&gt;</td>
</tr>
<tr>
<td>Haemodynamic parameters</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart rate (b.p.m.)</td>
<td>592 ± 22</td>
<td>556 ± 23</td>
<td>560 ± 13</td>
<td>534 ± 20</td>
</tr>
<tr>
<td>Systolic aortic pressure (mmHg)</td>
<td>118 ± 4.2</td>
<td>112 ± 3.3</td>
<td>220 ± 7.4&lt;sup&gt;1&lt;/sup&gt;</td>
<td>211 ± 6.3&lt;sup&gt;†&lt;/sup&gt;</td>
</tr>
<tr>
<td>LVEDP (mmHg)</td>
<td>2.84 ± 0.56</td>
<td>3.97 ± 0.61</td>
<td>5.60 ± 0.31&lt;sup&gt;1&lt;/sup&gt;</td>
<td>9.27 ± 0.60&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Maximum dp/dt (mmHg/s)</td>
<td>18.320 ± 510</td>
<td>17.100 ± 1030</td>
<td>15.200 ± 1180&lt;sup&gt;1&lt;/sup&gt;</td>
<td>9.950 ± 640&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Minimum dp/dt (mmHg/s)</td>
<td>−10,540 ± 440</td>
<td>−10,960 ± 550</td>
<td>−11,080 ± 860</td>
<td>−8830 ± 390&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tau (ms)</td>
<td>7.55 ± 0.38</td>
<td>8.34 ± 0.43</td>
<td>9.57 ± 0.65&lt;sup&gt;t&lt;/sup&gt;</td>
<td>11.36 ± 0.49&lt;sup&gt;t&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

I<sub>V</sub>S, interventricular septal thickness; LVPW, left-ventricular posterior wall thickness; LVEDD, left-ventricular end-diastolic diameter; LVSD, left-ventricular end-systolic diameter; LVFS, left-ventricular fractional shortening; LVEDP, left-ventricular end-diastolic pressure.

Data are expressed as means ± SEM, n = 12 for each.

<sup>a</sup>P < 0.05, compared with sham operation in the respective genotype.

<sup>t</sup>P < 0.05, compared with GCLM<sup>+/+</sup>.

GCLM<sup>−/−</sup> than in GCLM<sup>+/+</sup> mice (Table 1). GCLM<sup>−/−</sup> mice had significantly higher LVEDP and depressed minimum dp/dt after TAC than GCLM<sup>+/+</sup> mice (Table 1). There were no significant differences in LVEDD, LVSDs, and LVFS after TAC between heterozygous knockout (GCLM<sup>−/+</sup>) mice and GCLM<sup>−/−</sup> mice (see Supplementary material online, Figure S1).

### 3.3 Fibrogenesis in myocardium and cultured mouse cardiac fibroblasts and microvessel density in myocardium

Masson’s trichrome staining showed that the increase in myocardial interstitial and perivascular fibrosis after TAC was greater in GCLM<sup>−/−</sup> than in GCLM<sup>+/+</sup> mice (Figure 1D–F). The increase in Smad3 phosphorylation and mRNA expression of connective tissue growth factor (CTGF), procollagen I and III, and transforming growth factor (TGF)-β2 after TAC were greater in GCLM<sup>−/−</sup> than in GCLM<sup>+/+</sup> mice (Figure 2A–E). The increase in myocardial expression and activity of MMP-2 after TAC was greater in GCLM<sup>−/−</sup> than in GCLM<sup>+/+</sup> mice (Figure 2G and H). Similarly, cultured fibroblasts from GCLM<sup>−/−</sup> hearts had a greater fibrogenesis compared with fibroblasts from GCLM<sup>+/+</sup> hearts, and the increase in fibrogenesis in GCLM<sup>−/−</sup> fibroblasts was reversed by the addition of GSH-EE in the culture medium (see Supplementary material online, Figure S2). Microvessel density in myocardium after TAC was lower in GCLM<sup>−/−</sup> than in GCLM<sup>+/+</sup> mice (Figure 1G and H, Supplementary material online, Figure S3C).

### 3.4 SERCA2a and Ca<sup>2+</sup> uptake in myocardium and cell shortening and [Ca<sup>2+</sup>], transient in cultured mouse cardiomyocytes

GCLM<sup>−/−</sup> myocardium had a suppressed SERCA2-dependent Ca<sup>2+</sup> uptake and a lower expression of SERCA2a mRNA after either sham operation or TAC compared with GCLM<sup>+/+</sup> myocardium (Figure 3A and B). Immunoblotting of the immunoprecipitated SERCA2a using an anti-GSH antibody showed that glutathionylation of SERCA2a was increased after TAC in both genotypes, but the increase was less in GCLM<sup>−/−</sup> than in GCLM<sup>+/+</sup> myocardium (Figure 3C). In cultured cardiomyocytes, GCLM<sup>−/−</sup> cardiomyocytes had a lower cell shortening and peak [Ca<sup>2+</sup>], transient in response to electrical stimulation and a more prolonged time to 50% decay of the [Ca<sup>2+</sup>], transient compared with GCLM<sup>+/+</sup> cardiomyocytes (Figure 3D–F, Supplementary material online, Figure S3D). The addition of GSH-EE in the culture medium reversed these abnormal responses of GCLM<sup>−/−</sup> cardiomyocytes (Figure 3D–F).

### 3.5 GSH levels, expression of GCLC and GCLM, antioxidant enzymes, and β myosin heavy chain in the myocardium after TAC

Myocardial GSH content after TAC in GCLM<sup>−/−</sup> mice was 31% of that in GCLM<sup>+/+</sup> mice (Figure 4A). Myocardial GSH content 4 weeks after TAC was lower than that after sham operation in both genotypes. Myocardial GSSG content after either sham operation or TAC was lower in GCLM<sup>−/−</sup> than in GCLM<sup>+/+</sup> mice.
Figure 1 Comparison of Kaplan–Meier survival curves (A), heart weight/body weight (HW/BW) ratio (B), lung weight/body weight (LW/BW) ratios (C), myocardial fibrosis (D–F), and microvessel density (G and H) after TAC between GCLM\(^{+-}\) and GCLM\(^{++}\) mice. (D–F) Myocardial fibrotic area was measured by analysis of digital images of sections stained by Masson’s trichrome and their representative images. (G and H) Ratio of number of microvessels per cardiomyocytes and representative images. Vessels are stained in red (Lectin-I staining), Cell surface membrane in green [wheat germ agglutinin (WGA) staining]. Scale bars in (E), (F), and (H) = 1 mm, 50 μm and 20 μm, respectively. \(n = 10\) in each experiment (B–D and G). \(\dagger P < 0.05\) compared with the respective sham-operated genotype. \(* P < 0.05. \ +/+\ denotes GCLM\(^{++}\) mice and \(-/-\ denotes GCLM\(^{+-}\) mice.
activity was lower in GCLM than in GCLM$^{+/+}$ myocardium (Figure 3G).

**Figure 2** Smad3 phosphorylation, expression of CTGF, procollagen I, and II in myocardium after TAC. (A) Smad3 phosphorylation. Upper panel shows representative blotting. (B–G) mRNA expression. The levels were normalized to GAPDH mRNA expression. (H) MMP-2 activity. Upper panel shows a representative zymogram. n = 6 in each experiment. *P < 0.05 compared with the respective sham-operated genotype. **P < 0.001. The data are expressed relative to the sham-operated GCLM$^{+/+}$ myocardium (=1).

**3.6 Oxidative stress in myocardium and mouse cardiac fibroblasts**

The rate of ESR signal decay of the hydroxy-TEMPO radical adducts in myocardial homogenates was increased 1 and 4 weeks after TAC compared with sham-operated mice in both genotypes, and the signal decay 1 week after TAC was greater in GCLM$^{-/-}$ than in GCLM$^{+/+}$ myocardium (Figure 5G). Similarly, there was a greater increase in HPF fluorescence intensity after TGF-B1 incubation of cultured cardiac fibroblasts from GCLM$^{-/-}$ than from GCLM$^{+/+}$ hearts, and this was reversed by GSH-EE (Figure 5H).

**3.7 Effects of GSH-EE supplementation during TAC in GCLM$^{-/-}$ mice**

Heart rate and systolic blood pressure at the aortic root 4 weeks after TAC in GCLM$^{-/-}$ mice were similar comparing GSH-EE and placebo treatment (see Supplementary material online, Figure S4A and B). GSH-EE treatment lowered LVd, LVd, the myocardial fibrotic area, LVEDP and tau, and improved LVFS, and minimum and maximum dp/dt after TAC in GCLM$^{-/-}$ mice (Figure 6A–D, Supplementary material online, Figure S4C–F). In addition, GSH-EE treatment increased microvessel density, glutathionylation of SERCA2a, and SERCA-dependent Ca$^{2+}$ uptake in myocardium after TAC in GCLM$^{-/-}$ mice (Figure 6E–G, Supplementary material online, Figure S3E). Compared with placebo, GSH-EE supplementation increased the myocardial content of GSH and GSSG after TAC in GCLM$^{-/-}$ mice (Figure 6H and I).

**3.8 Relationship between GCLM gene polymorphism and DCM in human subjects**

The genotype distribution (Table 2) was in Hardy–Weinberg equilibrium within control subjects and DCM patients (P = 0.34 and 0.35, respectively, using a χ² test). The T allele was more prevalent in patients with DCM than in control subjects (CT and TT genotypes: 36.1% in DCM vs. 18.6% in controls; P < 0.001). Multiple logistic regression analysis showed that the presence of the T allele was a significant risk factor for DCM that was independent of age, smoking, diabetes, systolic blood pressure, body mass index, or serum uric acid (OR 3.13, 95% CI: 2.28–4.44; P < 0.0001, Table 3).

**4. Discussion**

Myocardial GSH levels after TAC in GCLM$^{-/-}$ mice were only 31% of those in GCLM$^{+/+}$ mice. Pressure overload due to TAC induced greater LV dilation and dysfunction, and lung congestion in GCLM$^{-/-}$ than in GCLM$^{+/+}$ mice. These adverse responses of the heart to pressure overload may have partly contributed to lower survival in GCLM$^{-/-}$ mice compared with GCLM$^{+/+}$ mice. In addition, TAC induced greater fibrosis, lower SERCA Ca$^{2+}$ uptake, and higher βMHC switching in GCLM$^{-/-}$ than in GCLM$^{+/+}$ myocardium. These changes may partially account for the greater myocardial contractile dysfunction in GCLM$^{-/-}$ mice compared with GCLM$^{+/+}$ mice. Moreover, GSH-EE supplementation reversed these adverse phenotypic changes in the LV after TAC in GCLM$^{-/-}$ mice, and they were associated with an increase in myocardial GSH content. Thus, GSH depletion exacerbated LV remodelling, dysfunction, and fibrosis in the pressure-overloaded heart in GCLM$^{-/-}$ mice. In agreement with these in vivo studies, cardiac fibroblasts cultured from...
GCLM−/− mice had increased expression of profibrogenic molecules, and cultured cardiomyocytes had impaired [Ca2+]i transients and cell shortening. Furthermore, these changes in vitro were reversed by GSH-EE in cells cultured from GCLM−/− mice. Consistent with these experimental data, patients with DCM had a higher prevalence of −588T polymorphism of the GCLM gene than control subjects, although there may have been selection bias of both the DCM patients and control subjects because of a case–control design. Our previous study showed that patients with −588T polymorphism had a 40% reduction in plasma GSH levels compared with those with the CC genotype.11 Taken together, these experimental and clinical results suggest that chronic depletion of GSH might increase the susceptibility of human and murine hearts to cardiomyopathy.

The ESR study showed that there was greater generation of myocardial oxygen radicals after 1 week of TAC in GCLM−/− than in GCLM+/− mice. In cultured cardiac fibroblasts, the reversal of the cell phenotype by GSH-EE supplementation was accompanied by a parallel decrease in oxidative stress determined using HPF fluorescence intensity. These data indicate that increased oxidative stress may partially account for the greater adverse response of the heart to pressure overload in GCLM−/− mice than GCLM+/− mice. Despite a persistent decrease in myocardial GSH content in GCLM−/− mice, a compensatory upregulation of antioxidant enzymes including MnSOD and catalase in GCLM−/− mice may explain similar levels of myocardial oxygen radicals (as assessed by ESR) after 4 weeks of TAC in the two mouse genotypes. An increase
in the ratio of GSH/GSSG usually reflects an improvement of the redox state, but it was higher in GCLM \textsuperscript{-/-} than GCLM \textsuperscript{+/+} myocardium after TAC. The increased activity of glutathione reductase may, at least in part, participate in the higher ratio of GSH/GSSG in GCLM \textsuperscript{-/-} than GCLM \textsuperscript{+/+} myocardium. In this context, the GSH/GSSG ratio may not be useful as an indicator of redox status in heats of GCLM \textsuperscript{-/-} mice, because GCLM \textsuperscript{-/-} mice had a tendency of paradoxical reduction in myocardial content of GSSG in response to oxidative stress in addition to severe reduction of GSH production.

In a previous study that developed a mouse model of spontaneous cardiomyopathy due to cardiac-specific overexpression of hR120GCryAB, myocardial GSH levels increased by 30\% of normal by an increase in glutathione reductase activity due to upregulation of glucose-6-phosphate dehydrogenase.\textsuperscript{6} This report first showed that the reductive stress induces ventricular dilation and premature death on the basis of the increase in GSH concentrations.\textsuperscript{6} In contrast, the present study showed that a decrease in myocardial GSH by 70\% of normal levels was associated with ventricular dilation and dysfunction in response to pressure overload in GCLM \textsuperscript{-/-} mice. Thus, results of this previous study\textsuperscript{6} and the present study suggest that myocardial GSH levels need to be regulated within a narrow range to prevent cardiac dysfunction.

TGF-\(\beta\) may play a role in the pathogenesis of the myocardial fibrosis induced by pressure overload.\textsuperscript{2,16} Furthermore, GSH antagonizes TGF-\(\beta\)-induced fibrogenesis.\textsuperscript{17} Consistent with these previous reports, the present study showed that the increased expression of fibrogenic molecules and greater activation of Smad3 in myocardium after TAC in GCLM \textsuperscript{-/-} mice were reversed by GSH-EE treatment.

Since TGF-\(\beta\) can suppress GCLC expression,\textsuperscript{9,17} the increased expression of TGF-\(\beta\)2 may prevent upregulation of GCLC mRNA.

Figure 4  Content of GSH and GSSG, mRNA expression of GCLC and GCLM and GCL activity in myocardium. (A–C) GSH content, GSSG content, and GSH/GSSG ratio in myocardium, respectively. (D and E) Myocardial mRNA expression of GCLC and GCLM, respectively. The expression levels were normalized to GAPDH mRNA expression and were shown relative to the levels in sham-operated GCLM \textsuperscript{+/+} myocardium (=1). (F) GCL activity. \(n=6\) in each experiment. *\(p<0.05\) compared with the respective sham-operated group.
GCLM^2/2 myocardium after 4 weeks of TAC had greater expression and activity of MMP-2 than GCLM^+/+ myocardium. In addition, the present study showed low microvessel density in GCLM^2/2 myocardium after TAC. These may partly account for the increased ventricular dilation in GCLM^2/2 mice. It is known that many molecular mechanisms may regulate angiogenesis in pressure-overloaded heart. Although GSH supplementation reversed low microvessel density in GCLM^2/2 myocardium, the exact mechanisms for the impaired angiogenesis in GCLM^2/2 myocardium are unclear.

It is known that SERCA activity is reversibly increased as its glutathionylation increases. The present study showed that SERCA2a was glutathionylated after TAC to a lesser extent in GCLM^2/2 than GCLM^+/+ myocardium. The exact mechanisms for the low glutathionylation of SERCA2a in GCLM^2/2 are not clear. However, it is known that protein glutathionylation can occur by several reactions, including direct interaction between partially oxidized protein sulfhydryls and GSH, or thiol/disulfide exchange reactions between protein thiols and GSSG. Thus, profound decrease in GSSG as well as GSH content may at least partly contribute to the lower SERCA2a glutathionylation in GCLM^2/2 than GCLM^+/+ mice, because GSH supplementation reversed the SERCA2a glutathionylation in parallel with an increase in myocardial levels of GSSG and GSH in GCLM^2/2 mice. In addition, SERCA2a expression was decreased after TAC more in

Figure 5 Activities of antioxidant enzymes, expression of αMHC and βMHC in myocardium and cultured mouse cardiac fibroblasts. (A–C) Myocardial enzymatic activities of glutathione reductase (GR), catalase, and MnSOD, respectively. (D–F) Myocardial expression of mRNA of βMHC and α-myosin heavy chain (αMHC) and the βMHC/αMHC ratio, respectively. (G) The rate of ESR signal decay of hydroxy-TEMPO in myocardial homogenates. The rate was normalized to that in sham-operated myocardium in GCLM^+/+ mice (=1). (H) Intracellular oxygen radicals in cultured cardiac fibroblasts after stimulation with TGF-β1 (25 ng/mL) in the presence or absence of GSH-EE (2 mmol/L) was assessed by HPF intensity. HPF intensity values were calculated after subtracting background fluorescence, and normalized the values to fluorescence levels before TGF-β1 or vehicle addition. n = 6–8 in each experiment. *P < 0.05 compared with the respective sham-operated group or vehicle-treated cells. †P < 0.05.
Table 2 Genotypes frequencies of GCLM promoter polymorphism in control subjects and patients with DCM

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Control subjects (n = 253)</th>
<th>DCM patients (n = 205)</th>
<th>OR (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>−588C/T</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>−588TT</td>
<td>1/253 (0.4)</td>
<td>11/205 (5.4)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>−588CT</td>
<td>46/253 (18.2)</td>
<td>63/205 (30.7)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>−588CC</td>
<td>206/253 (81.4)</td>
<td>131/205 (63.9)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>−588T vs. C allele</td>
<td>—</td>
<td>—</td>
<td>2.50 (1.70–3.65)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>−588TT and CT vs. CC</td>
<td>—</td>
<td>—</td>
<td>2.48 (1.62–3.79)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>−588TT vs. CT and CC</td>
<td>—</td>
<td>—</td>
<td>14.3 (1.8–111.6)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Data presented are number of patients (%). CI, confidence interval; OR, odds ratio; DCM, dilated cardiomyopathy.

*Fisher’s exact probability.
GCLM<sup>–/–</sup> than in GCLM<sup>+/+</sup> myocardium. Thus, the lower glutathionylation and expression of SERCA2a may contribute to a lower SERCA-dependent Ca<sup>2+</sup> uptake after TAC in GCLM<sup>–/–</sup> myocardium than in GCLM<sup>+/+</sup> myocardium.

There were several limitations of this study. First, the timepoint of harvesting the heart after the last GSH-EE injection may influence the expression of SERCA2a glutathionylation and myocardial GSH and GSSG content, because the increase in myocardial GSH content after GSH-EE injection is transient.<sup>21</sup> Secondly, this study did not examine effects of GSH-EE supplementation on all adverse phenotypes after TAC in GCLM<sup>–/–</sup> mice because of a limited supply of a large amount of GSH-EE which was required for long-term supplementation.

In conclusion, chronic depletion of GSH was associated with LV dilatation, fibrosis, and dysfunction after pressure overload in mice deficient of GCLM. The clinical relevance of this mouse study is supported by the human study showing that –588T polymorphism of the GCLM gene was associated with idiopathic DCM.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

Acknowledgements

We gratefully acknowledge the technical assistance of A. Watanabe and H. Watanabe.

Conflict of interest: none declared.

Funding

This study was partly supported by the Ministry of Education, Culture, Sports, Science and Technology, Health, Tokyo, Japan (grants-in-aid for B2-19390209 and B-22390158).

References