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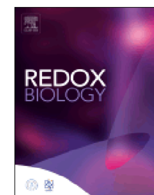

Research Paper

Glutathione inhibits antibody and complement-mediated immunologic cell injury via multiple mechanisms

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A B S T R A C T

Antioxidant glutathione (GSH) plays an important role in the regulation of immunity. However, little is known about its effects on humoral immunity, especially its action on effector molecules like antibody and complement. Given that these molecules contain abundant disulfide bonds, we speculated that GSH might influence the action of these proteins via its thiol function. Using a model of a glomerular mesangial cell (MC) lysis induced by antibodies plus complement, we addressed this hypothesis. Exposure of rat MCs to anti-Thy-1 antibody plus complement or anti-MC rabbit serum caused a complement-dependent cell lysis, which was completely blocked by GSH. Moreover, GSH potently prevented the antibody-mediated agglutination of red blood cells and aggregation of antibody-sensitized microspheres. Further analysis revealed that GSH inhibited antibody binding to antigens and promoted the conversion of the antibodies to its reduced forms. GSH also potently inhibited the formation and deposition of C5b-9 in MCs and suppressed both the classic and alternative complement activation pathway. Lastly, GSH attenuated P38 activation, an oxidative sensitive kinase that partially mediated the antibody- and complement-dependent MC lysis. Depletion of GSH via inhibiting gamma-glutamylcysteine synthetase or xCT transporter augmented P38 activation and sensitized MCs to the cell lysis. Collectively, our results indicate that GSH protects cells from immunological cell damage via mechanisms involving inhibition of antibody binding to the antigens, suppression of complement activation and augmentation of cellular defense mechanism. Our study provides novel mechanistic insights into the actions of GSH in the regulation of immune responses and suggests that GSH might be used to treat certain immune disorders.

1. Introduction

The tripeptide glutathione (γ-l-glutamyl-l-cysteinyl-glycine, GSH) is the most abundant low molecular weight thiol-containing peptide within the cells (intracellular concentration up to 10 ~ 15 mM). It exerts many biological functions. Most of them are attributable to its thiol function. GSH scavenges free radical and reactive oxygen species and facilitates the regeneration of other antioxidants. It is the major cellular defense mechanism against oxidative stress. Besides, GSH also serves as a key cofactor for many enzymes. Recently, GSH has been recognized to act not only as a major antioxidant within the cells, but also as a mediator of many other physiologic reactions, including metabolism of xenobiotics, thiol-disulfide exchange reactions, and cellular signaling. Dysfunction of GSH contributes to the initiation and development of many diseases [1].

GSH has been documented to regulate immune responses and has been shown to be critically implicated in the inflammatory diseases. GSH deficiency is one of the major pathogenic factors leading to the oxidative and immune diseases. Supplementation of GSH has been reported to be effective in prevention or alleviation of many immune-related diseases [1,2]. GSH regulates several pivotal molecular events involved in immune responses. For examples, GSH affected the activation of NF-κB [3–6], a transcription factor that plays a key role in the inflammation. It also potently suppressed inflammasome activation, a molecular event that links oxidative stress, inflammation and cell injury [7–9]. Furthermore, GSH modulated macrophage phagocytosis [10,11] and T cell receptor-mediated signal transduction. It stimulated IL-2 production, promoted T cell proliferation, and enhanced T cell resistance to apoptosis [12,13]. The mechanisms involved are thought to be related to its regulation on intracellular redox status and redox

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signaling.

Most of the studies regarding the regulatory effects of GSH on immunity have been limited to cell immunity. Our understanding about its roles on humoral immunity are still limited. In this aspect, GSH has been reported to selectively decrease IL-4-induced immunoglobulin (Ig) E and IgG4 production by blood mononuclear cells in vitro and decreased both IgE and IgG1 antibody production. In addition, in vivo administration of GSH precursor NAC decreased both IgE and IgG1 antibody responses to ovalbumin [14]. GSH has been reported to regulate IgE isotype switching via inhibition of NF-κB in cultured B cells [12]. NAC also significantly suppressed the specific antibody response via regulation of the functional molecules in B cells, as well as the production of IL-1 and IFN-γ [15]. Currently, few studies are available concerning the direct effect of GSH on soluble effectors of humoral immunity like antibodies and complement. Given that most of these proteins contain abundant disulfide bonds and that these bridges are indispensable for the maintenance of the normal structure and function [16,17], we speculated that GSH might be a critically involved in the regulation of these molecules. This study was designed to address this hypothesis.

Using an in vitro model of antibody and complement-initiated cell lysis, we investigated the potential effects and mechanisms of GSH on antibody-initiated and complement-dependent cell injury. Here we present our results showing that GSH potently inhibited antibody- and complement-initiated immune responses through mechanisms involving its action on antibody, complement, and cell defense. Our study thus indicates that GSH potently regulates humoral immune responses and suggests that GSH has the potential to treat certain immune-mediated diseases.

2. Materials and methods

2.1. Materials

Calcein AM was obtained from Invitrogen (Tokyo, Japan). Dynabeads protein A and protein G Mag Sepharose were from Novex in life technologies (Life Technologies-Novex, Carlsbad, CA) and GE Healthcare (UK), respectively. HRP-conjugated anti-rabbit or mouse IgG and anti-β-actin antibodies were purchased from Cell Signaling, Inc. (Beverly, MA). Anti-Thy-1 monoclonal antibody 1–22-3 was kindly gifted by Dr. Kawachi (Institute of Nephrology, Niigata University). BSO was obtained from Cayman Chemical (Michigan, USA). Rhodamine-conjugated anti-rabbit IgG antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-mouse red blood cell (RBC) antibody was purchased from Abgent (San Diego, CA). The Easy-Titer IgG Assay Kit was purchased from Thermoscientific (Rockford, IL). The Enzyme immunoassay for assessment of complement functional activity kit was purchased from Wieslab (Malmö, Sweden). GSH and all other reagents were bought from Sigma (Tokyo, Japan).

2.2. Rabbit serum

Rabbit blood was collected from ear aricular artery of the Japanese White Strain Rabbit. The blood was allowed to clot for 30 min at room temperature. The serum was collected and stored at −80 °C until use. For inactivation of complement, the serum was heated at 56 °C for 30 min. All animal experiments were approved by the animal experiment committee of the University of Yamanashi and performed following the relevant guidelines and regulations.

2.3. Cells

Rat glomerular mesangial cells (MCs) were obtained from the outgrowth of the isolated glomeruli, as described previously [18,19]. Cells were cultured in DMEM (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 5% FBS for passage and expansion. For experiments, cells were cultured with DMEM containing 1% FBS with or without the presence of various stimuli.

2.4. Calcein-AM and propidium Iodide (PI) staining

Cell viability was determined by the Calcein-AM/PI live/dead staining as described previously [18]. Living cells exhibit positive staining for calcein-AM activity (green fluorescence), whereas dead cells stain positive for PI (red fluorescence).

2.5. Precipitation of membrane-bound antibodies

Immunoglobulins (Igs) bound to the cell membrane were precipitated using a mixture of protein A and G beads as reported previously [18]. MCs were reacted with rabbit serum or antibodies for the indicated period. After washing out the unbound free Igs with PBS for two times, total cellular proteins were extracted by suspending the cells in RIPA lysis buffer. Lysates were incubated on ice for 15 min with intermittent mixing and then centrifuged at 15,350 × *g* for 10 min at 4 °C. The supernatant was recovered and determined for protein concentration using the Micro BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA). Same amount of lysate in 300 µl RIPA was incubated with a mixture of protein A and G beads in a rotator at 4 °C overnight. The pellet was washed with 1 ml RIPA for three times and resuspended in 50 µl 2.5 X SDS sample buffer containing five mM DTT. After heat treatment at 95–100 °C for 5 min, supernatants were collected and loaded on a 10% gel for SDS-PAGE. The separated proteins were transferred to PVDF membrane and immunoblotted for cell bound-Igs.

2.6. Lactate dehydrogenase (LDH) release assay

Cell viability was evaluated by the release of LDH using an LDH cytotoxicity detection kit (Takara Bio, Inc., Otsu, Shiga, Japan). Briefly, cells in 96-well culture plate were exposed to various stimuli for the indicated time intervals. Culture medium was collected and added to wells at the volume of 30 µl. After reaction with the same volume of assay solution, the optical absorbance of the red color formed in the assay was measured at a wavelength of 490 nm with a UV–VIS spectrophotometer. LDH activity was calculated and expressed as a percentage of 100% whole release as made by exposing cells to Triton X-100.

2.7. Assessment of cell viability with WST reagent

Cells were seeded into 96-well culture plates and exposed to various stimuli in the presence or absence of GSH. WST reagent was added into each well 2 h before measurement of OD with a spectrometer at the wavelength of 450 nm [20].

2.8. Immunofluorescence staining

For immunofluorescence staining of membrane-bound IgG, mesangial cells were pretreated with 1% heat-treated rabbit serum in the presence or absence of the indicated concentration of GSH for 1 h. The cells were then rinsed with PBS, fixed with 3% paraformaldehyde, and stained with tetramethy1 rhodamine B isothiocyanate-conjugated anti-rabbit immunoglobulin G for 1 h. After washed with PBS, cells were observed under IF microscopy and positive IF signals in MCs were captured using a CCD camera attached to an Olympus BX50 microscope. For assessment of C9 deposition, MCs were treated with 10 µg/ml Thy-1 plus 10% human serum as a source of complement in the presence or absence of 5 mM GSH for 30 min. After washing and fixation as described above, cells were incubated with an anti-human C9 antibody at room temperature for 2 h, followed by a further step of washing and incubation with tetramethy1 rhodamine B isothiocyanate-

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