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Glyoxalase I activity and immunoreactivity in the aging human lens

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Abstract

Glyoxalase I (GLOI) is the first enzyme of the glyoxalase system that catalyzes the metabolism of reactive dicarbonyls, such as methylglyoxal (MGO). During aging and cataract development, human lens proteins are chemically modified by MGO, which is likely due to inadequate metabolism of MGO by the glyoxalase system. In this study, we have determined the effect of aging on GLOI activity and the immunoreactivity and morphological distribution of GLOI in the human lens. A monoclonal antibody was developed against human GLOI. GLOI immunoreactivity was strongest in the anterior epithelial cells and weaker in rest of the lens. Cultured human lens epithelial cells showed immunostaining throughout the cytoplasm. In the human lens, GLOI activity and immunoreactivity both decreased with age. We believe that this would lead to promotion of MGO-modification in aging lens proteins.

Keywords

Glyoxalase I; Monoclonal antibody; Human lens; Aging; Enzyme activity; Enzyme immunoreactivity

Introduction

The glyoxalase system consists of two enzymes; glyoxalase I (GLOI) and glyoxalase II (GLOII), which catalyze the metabolism of reactive α -dicarbonyls such as methylglyoxal (MGO) and glyoxal (GO) (Mannervik 2008; Silva et al. 2008; Thornalley 2003). In MGO metabolism, GLOI catalyzes the conversion of hemithioacetal (generated in the non-enzymatic reaction of glutathione with MGO) to lactoylglutathione. This product is then converted to D-lactate by GLOII, and glutathione is released. MGO is mostly produced from the glycolytic intermediates, glyceraldehyde-3-phosphate and dihydroxyacetone-phosphate, through β -elimination of the phosphate group and dehydration, whereas GO is produced during lipid and sugar oxidations (Thornalley 2007).

Both MGO and GO are highly reactive molecules that can chemically modify cysteine, arginine and lysine residues in proteins (Degenhardt et al. 1998). Many arginine and lysine modifications have been identified, several of which have been detected in human and animal tissues, and the relationship of these modifications to diabetic complications has been proposed (Ahmed and Thornalley 2002; Biemel et al. 2002; Padayatti et al. 2001b; Shamsi et al. 1998; Wilker et al. 2001). It is generally believed that the reaction of MGO with proteins is harmful, in terms of protein structure and function; however, a few studies have shown beneficial effects, particularly on the chaperone function of human α A-crystallin (Kumar et al. 2004; Nagaraj et al. 2003).

Several studies demonstrate that GLOI is critical for removal of MGO to prevent its reaction with proteins (Miller et al. 2006; Shamsi et al. 2000). Overexpression of GLOI in human umbilical vein endothelial cells reduces intracellular MGO concentrations (Shinohara et al. 1998) and reverses hyperglycemia-induced defects in angiogenesis (Ahmed et al. 2008). Inhibition of GLOI using a specific chemical inhibitor increases the concentrations of MGO and MGO-derived products on proteins in retinal endothelial cells (Padayatti et al. 2001a). In addition, inhibition of GLOI sensitizes human retinal pericyte for apoptosis under in vitro conditions of high glucose (Miller et al. 2006). These studies implicate GLOI in macro- and microvascular complications in diabetes. GLOI activity is also related to aging as its overexpression causes life-span extension in *C.elegans* (Morcos et al. 2008). It has also been observed that GLOI activity declines with aging in skeletal muscles and brain, which could lead to reduced metabolism of MGO and thus protein modifications in these tissues (Piec et al. 2005).

GLOI has also been implicated in other diseases and disorders. GLOI content has been shown to decrease in Alzheimer's disease-afflicted brains in humans (Kuhla et al. 2007), that could lead to enhanced MGO-modification of proteins contributing to pathology of the disease. In autism, a GLOI single nucleotide polymorphism (possibly leading to loss of GLOI activity) and MGO-modifications were potentially involved (Junaid et al. 2004), although a recent study has questioned this finding (Rehnstrom et al. 2008). GLOI overexpression has been shown to cause enhanced anxiety-like behavior in mice (Hovatta et al. 2005). Furthermore, malignant cells in cancer appear to express high levels of GLOI (Rulli et al. 2001), possibly to remove MGO formed upon enhanced metabolism. A recent study indicated that GLOI is necessary for osteoclastogenesis (Kawatani et al. 2008), that plays a role in remodeling of bone matrix.

Proteins of the aging human lens accumulate many post-synthetic modifications due to their negligible turnover. There is compelling evidence for protein modifications by MGO in the aging lens. In general, aged lenses show higher levels of MGO-modifications than young lenses, and such modifications further increase in cataractous lenses (Ahmed et al. 2003; Biemel et al. 2002; Shamsi et al. 1998; Wilker et al. 2001). Both GLOI and GLOII activities

have been observed in the human lens (Haik et al. 1994). Based on MGO-modifications in aging and cataractous lenses, it can be speculated that GLOI activity is decreased during lens aging and cataract formation. In the present study, using a monoclonal antibody that we developed, we demonstrate that both the enzyme activity and immunoreactivity are decreased in aging lenses.

Materials and methods

Cloning, expression and purification of human GST-GLOI and his-GLOI

GST-GLOI was amplified from human GLOI in the pUC19 backbone (kindly provided by Dr. Sulabha Ranganathan). PCR was carried out with forward primer (5'-CAT GCC ATG GCA GAA CCG CAG CCC CCG-3') and reverse primer (5'-CCC AAG CTT CTA CAT TAA GGT TGC CAT TTT-3'). The amplified GLOI was inserted into the pET-41a(+) vector using the NcoI and HindIII restriction sites. pET-41a(+) had an N-terminal GST and 6XHis-tag. The N-terminal 6XHis-tag was introduced by PCR into human GLOI using forward primer (5'-CAT GCC ATG GGG CAC CAC CAC CAC CAC ATG GCA GAA CCG CAG CCC CCG-3') and reverse primer (5'-CCC AAG CTT CTA CAT TAA GGT TGC CAT TTT-3'). The amplified GLOI was inserted into the pET23d(+) vector using NcoI and HindIII restriction sites.

BL21 (DE3)pLysS bacteria were transformed with the GLOI-pET-41a(+) plasmid to express GST-GLOI fusion protein. Since the plasmid also had an N-terminal His tag, Ni-NTA resin was used for protein purification. LB broth (1 l) containing 50 lg/ml kanamycin was inoculated with 50 ml of an overnight culture of BL21 (DE3)pLysS expressing GST-GLOI fusion protein and cultured at 37°C, 250 rpm until the optical density (OD) at 600 nm was 0.5–0.7. Induction was done using 100 mM IPTG for 5 h at 37°C. Cells were harvested by centrifugation at 5,000g for 15 min at 4°C. The cell pellet was resuspended in 5 ml/gm cell pellet of Cell Lytic B Cell Lysis Reagent (Sigma) and gently mixed for 2 h at RT. The sample was centrifuged at 16,500g for 15 min, and the supernatant was incubated overnight at 4°C with an appropriate amount of Ni-NTA resin. The column was washed and eluted according to manufacturer's instructions (Qiagen, Inc., Valencia, CA). The fractions showing GLOI activity were pooled, dialyzed against PBS for 24 h and concentrated using Amicon Ultra-15 centrifugal filters (Millipore) and stored at –20°C. This preparation showed both GLOI and GST in liquid chromatography/mass spectrometry analysis.

BL21 (DE3)pLysS bacteria were transformed with GLOI-pET-23d(+) plasmid to express the N-terminal 6XHis-tagged GLOI protein (henceforth referred as his-GLOI). LB broth (1 l) containing 100 lg/ml ampicillin was inoculated with 50 ml of an overnight culture of BL21 (DE3)pLysS expressing N-terminal 6XHis-tagged GLOI. The protein was purified using Ni-NTA resin under native conditions as per manufacturer's instructions.

Production of a monoclonal antibody against human GLOI

All animal experiments conformed to the ARVO Statement on the use of animals in Ophthalmic and Vision Research and approved by the Case Western Reserve University's Institutional Animal Care and Use Committee. BALB/c mice were immunized by intraperitoneal (i.p.) injection of 30 lg recombinant human GLOI-GST fusion protein in PBS emulsified with an equal volume of complete Freund's adjuvant, followed by three i.p. booster injections of 10 lg of protein in incomplete Freund's adjuvant. Blood samples were collected from the tail vein 20 days after the final injection. Serial dilutions of sera were tested by enzyme-linked immunosorbent assay (ELISA; see below) with GLOI as the coating protein. The mouse which had the highest immunoreactivity on ELISA was injected intravenously with 30 lg of immunogen in PBS 3 days before the fusion process.

Spleen cells were fused with murine myeloma cells SP2/O using polyethylene glycol (Oya et al. 1999), and the hybridomas were cultured in selection medium containing hypoxanthine, aminopterin and thymidine. By an ELISA (as described below), we selected clones that were highly reactive to his-GLOI, but not to GST. The clones were then subcloned by limited dilution. We determined the IgG subclass using an IsoStrip Kit (Roche Diagnostics Corporation, Indianapolis, IN). Three monoclonal hybridomas, GLOIa, GLOIb and GLOIc, were found to be reactive with hGLOI, of which GLOIa was found to be the most reactive. GLOIa was expanded in serum-free hybridoma medium (Roche Diagnostics Corp., Indianapolis, IN) for antibody production, and antibody was purified from 50 ml culture supernatant on Protein G-Sepharose (Invitrogen, CA).

Enzyme-linked immunosorbent assay

Microplate wells were coated overnight with 1 μ g protein/well in 50 mM carbonate buffer (pH 9.7). The wells were washed three times with PBS containing 0.05% Tween-20 (PBS-T) and blocked with 5% nonfat dry milk (NFDm) in PBS-T for 2 h at room temperature. The wells were then washed three times with PBS-T and incubated with 50 μ l GLOI mAb for 1 h at 37°C in a humidified chamber. The antibody was diluted in PBS. For hybridoma screening, we used 50 μ l culture supernatant. Following this step, the wells were washed three times with PBS-T and incubated for 1 h at 37°C with 50 μ l of goat anti-mouse IgG diluted in PBS-T (1:5,000). After washing three times with PBS-T, the wells were incubated with 100 μ l of 3,3',5,5'-tetramethylbenzidine substrate (Sigma). The enzyme reaction was quenched by addition of 50 μ l of 2 N H₂SO₄, and absorption was measured at 450 nm in a Dynex MRX 5000 Microplate Reader.

Western blotting

Proteins were electrophoresed by 15% SDS-PAGE and transferred onto nitrocellulose membrane (Bio-Rad, Hercules, CA). The membrane was blocked with 5% NFDm/PBS overnight at 4°C and then incubated for 1 h at 4°C with 10 ml of GLOI mAb diluted (12 ng/ml) in 5% NFDm/PBS. After washing five times (5 min each) with PBS-T, the membrane was incubated for 1 h at room temperature with a 1:5,000 dilution (in PBS-T) of goat anti-mouse IgG conjugated with horseradish peroxidase (Promega). After five washes (5 min each) with PBS-T, the membrane was treated with SuperSignal West Pico chemiluminescence substrate (Pierce) for 5 min and exposed to X-ray film (Pierce).

Immunohistochemistry

After rehydration, high temperature antigen retrieval and washing in cold PBS, the paraffin-embedded sections of human lens were blocked with goat serum (2.5% in PBS) for 2 h at RT. The sections were then incubated with purified monoclonal GLOI mAb (10 μ g/ml in PBS) for 1 h at RT and rinsed thoroughly with PBS. Sections were then incubated with goat anti-mouse IgG Texas Red (Molecular probes) at a 1:400 dilution in PBS for 1 h at RT, rinsed thoroughly with PBS, incubated with DAPI/Vectashield for 1 min and then mounted. Images were acquired as described above. To obtain the stitched images, Metamorph software (Molecular Devices Corp.) was used.

Immunocytochemistry

Human lens epithelial cells (HLE-B3 cell line from Dr. Usha Andley, University of Washington) were cultured for 1 day in chamber slides, then washed twice with PBS and fixed with 4% paraformaldehyde in PBS at -20°C for 15 min. After two PBS washes, they were permeabilized with 0.1% Triton X-100 in PBS at -20°C for 5 min. To remove the detergent, the cells were washed five times with PBS and then blocked with 2.5% goat serum in PBS for 2 h at RT. Cells were incubated with GLOI mAb (10 μ g/ml) in PBS for 1

h at RT and washed twice for 5 min with PBS. Cells were incubated with secondary antibody (anti-mouse IgG) conjugated with Texas Red (Molecular Probes) at a 1:400 dilution in PBS for 1 h at RT. After washing twice with PBS for 5 min, they were incubated with phalloidin (Invitrogen), prepared as per manufacturer's instructions, for 30 min at RT followed by two washes for 5 min in PBS. Cells were then incubated with DAPI/Vectashield for 1 min and permanently mounted. Lens images were acquired using a Leica DMI 6000 B inverted microscope with a 209 objective connected to a Retiga EXI camera (Q-imaging Vancouver British Columbia). Background due to the secondary Ab was verified by staining with a no primary Ab control.

Preparation of human lens extracts

Lenses from non-diabetic donors, obtained from the National Disease Research Interchange (Philadelphia, PA), were included in the study. Lenses were homogenized on ice in PBS containing proteinase inhibitor cocktail (Sigma). The insoluble fraction was removed by centrifugation at 10,000g at 4°C for 15 min, and the supernatants were collected. Protein concentration was determined by the Bio-Rad method using BSA as standard, and GLOI activity was estimated as described (see below).

Assay of GLOI activity

GLOI activity was determined spectrophotometrically (Miller et al. 2006) with 2.0 mM MGO and 2.0 mM GSH in 0.1 M sodium phosphate buffer, pH 7.0 at 25 ± 2°C in 1.0 ml. MGO and GSH were pre-incubated to form hemithiocetal. The formation of the S-D-lactoylglutathione was tracked at 240 nm. The absorption coefficient used for S-D-lactoylglutathione formation was 3.37 mM⁻¹ cm⁻¹. One unit of enzyme corresponds to 1.0 μmol of product formed per min/mg protein.

Results

Cloning, expression and purification of GST-GLOI and his-GLOI

The GST-GLOI chimeric protein had a molecular weight of ~53 kDa on SDS-PAGE, which corresponded to GLOI+ GST+ a 30 amino acid insert (present in the vector) in between GLOI and GST+ 6 histidine residues. The his-GLOI showed a molecular weight of ~23 kDa on SDS-PAGE (data not shown). Western blotting showed strong immunoreactivity with his-GLOI, GST-GLOI, but not with GST (Fig. 1a), suggesting that the purified antibody is specific to human GLOI and does not react with GST. The identity of GLOI (in GST-GLOI and his-GLOI) was further confirmed by LC-mass spectrometry of the trypsin-digested proteins (data not shown).

Production of monoclonal antibody

Three hybridomas, GLOIa, GLOIb and GLOIc, were found to secrete monoclonal antibody specific for hGLOI. Using IsoStrip Kit, we identified antibodies from GLOIa and GLOIb as IgG1 κ and that from GLOIc as IgG2a κ. We selected GLOIa mAb for subsequent experiments as it showed the highest reactivity against GLOI (data not shown). We purified the antibody from culture supernatants using Protein G-Sepharose (GE Health Sciences, Piscataway, NJ), as per manufacturer's instructions. The purified antibody was stored at -80°C. GLOIa, henceforth referred to as GLOI mAb, was used in the present work.

Characterization of monoclonal antibody

ELISA was performed using his-GLOI, GST-GLOI, and GST from human placenta (Sigma) as the coating proteins. The antibody showed immunoreactivity against his-GLOI and GST-GLOI, but not against GST (data not shown). GST coating to the ELISA plate well was

confirmed using mouse anti-human GST antibody (Sigma). Western blotting showed strong immunoreactivity with his-GLOI, GST-GLOI, but not with GST (Fig. 1a). These data show that the purified antibody is specific to human GLOI and does not react with GST. The optimum GLOI antibody concentration for ELISA was assessed using 0.075–120 ng antibody in 50 μ l/well (coated with 1.0 μ g protein of his-GLOI). We found that 0.15 ng of the antibody gave an OD of 0.9 within 30 min of incubation. Next, we determined the optimum his-GLOI concentration for coating in ELISA wells using 0.001–4.0 μ g protein his-GLOI/50 μ l. The antibody gave an OD of 0.4 within 30 min with 0.01 μ g his-GLOI (Fig. 1b). Based on these data, we decided to use 0.01 ng/well of his-GLOI for coating and 0.15 ng/well of the antibody for subsequent experiments.

Immunohistochemistry for GLOI in human lens and cultured human lens epithelial cells

To determine morphological distribution of GLOI in the human lens, paraffin-embedded lens sections were immunostained with GLOI mAb after high temperature antigen retrieval by microwave irradiation. High immunoreactivity was detected in the anterior epithelium, which coincided well with DAPI staining (Fig. 2a, experimental). Mild immunoreactivity was observed in the rest of the lens. Sections incubated without the primary antibody (control) showed low non-specific binding of the secondary antibody (red fluorescence) throughout the tissue section. Additional washing steps after incubation of the secondary antibody did not yield better results. This non-specific binding might be due high protein content in the lens. Nonetheless, higher fluorescence in the presence of the primary antibody and localization of intense fluorescence in the epithelium, together with data on cultured lens epithelial cells (see below), confirmed that the immunoreactivity was due to GLOI.

To further confirm high immunoreactivity in epithelial cells, we used cultured human lens epithelial cells (HLE-B3). Strong immunoreactivity was detected throughout the cytoplasm (Fig. 2b, experimental). Omission of the primary antibody (control) resulted in the absence of immunoreactivity, confirming that the immunoreaction is due to GLOI in cells. Enzyme activity measurement in the lysate of these cells showed ~70 units/mg protein, and the activity was inhibited when cell lysate was incubated with 20 μ M *S*-*p*-bromobenzylglutathione cyclopentyl diester, a competitive inhibitor of GLOI. Together, these data suggest that GLOI is mainly localized in the anterior epithelium but the enzyme is diffusely present in outer cortical and nuclear regions of the human lens.

GLOI activity and immunoreactivity in human lenses

GLOI activity was measured spectrophotometrically by monitoring the absorbance at 240 nm from *S*-*D*-lactoylglutathione, which is formed from GLOI catalyzed isomerization of hemithioacetal (formed from non-enzymatic reaction of MGO and GSH). Figure 3a shows traces from the enzyme assay using his-GLOI and human lens proteins. A time dependent increases in absorbance was observed and the enzyme activity was calculated from the slope of the curves. To study the effect of aging on GLOI in the human lens, we first determined the enzymatic activity. The study included lenses from donors of age between 8 and 80 years. Enzyme activity was detectable in all lenses. A specific activity of ~65 was observed in 8–20 year old lenses. However, it declined in lenses older than 32 years (Fig. 3b). For example, in a 32-year-old lens, the activity was 42% lower than in an 8-year-old lens, and it was further lowered to 82% in an 80-year-old lens. These data suggest that GLOI activity in the human lens is reduced due to aging. Together, these data suggested that GLOI is inactivated in the lens in the aged lens.

We calculated the concentration of GLOI in the human lens based on the immunoreactivity of his-GLOI in ELISA (Fig. 1b). In the young lenses (<30 years), the concentration was ~2–3 μ g/mg of protein and in aged (>60 years), the concentration was ~1–2 μ g/mg of protein.

We further investigated the GLOI immunoreactivity in aging lenses by a direct ELISA. ELISA plate wells were coated with 2.0 μ g of lens proteins in 50 μ l and probed with GLOI mAb. The results are shown in Fig. 4. The immunoreactivity was similar up to 20 years of age, but it declined in lenses older than 30 years. The immunoreactivity decreased by ~10% in a 32-year-old lens when compared to a 15-year-old lens, and it further decreased by ~30% in an 80-year-old lens. In addition, GLOI Western blotting results in three representative lenses of different ages is shown in Fig. 4 inset. The immunoreactivity was similar in 10 and 46-year-old lens and but was slightly decreased in the 73-year-old lens. The antibody pre-incubated with his-GLOI before addition to wells showed no reaction with lens proteins (data not shown). Together these data confirm that the GLOI content decreases in aging lenses.

Discussion

The purpose of this study was to determine the tissue distribution of GLOI and its association with enzyme activity in the aging human lens. To address this, we developed an antibody for human GLOI. Specifically, we generated a monoclonal antibody against a GST-GLOI chimeric protein, as GLOI is more stable and easier to purify when conjugated to GST. The purified protein had the expected molecular weight of ~53 kDa and contained both proteins as identified by mass spectrometry. The purified protein was used as the immunogen and hybridomas were screened for reaction with both GST and GLOI, and the GLOI-only reacting hybridomas were then selected for antibody purification. Our subsequent studies revealed that we could purify 6XHis-conjugated GLOI (at the N-terminus) with high yield and activity. The purified protein was verified by mass spectrometry, which showed the expected molecular weight. The final antibody did not react with GST but reacted strongly with both GST-GLOI chimeric protein and his-GLOI.

The monoclonal antibody enabled us to make the following observations: (1) in the human lens, GLOI is mainly present in the anterior epithelium, and diffusively in rest of the lens, (2) GLOI immunoreactivity and activity decrease in the aging human lens and (3) GLOI is likely to be chemically modified resulting in its inactivation.

It is not surprising that, like major anti-oxidative enzymes (Fujiwara et al. 1992; Mancini et al. 1989; Reddan et al. 1996), most of GLOI is localized in the epithelium, as this is the most metabolically active part of the lens (Bhat 2001). It is likely that much of MGO is produced here and that it might require high levels of GLOI for metabolism. This is supported by the observation of high immunoreactivity throughout the cytoplasm of cultured human lens epithelial cells. The high content of GSH in the epithelium, which is essential for GLOI activity, supports the requirement for high amounts of GLOI for MGO metabolism. Furthermore, for complete metabolism of MGO, the glyoxalase system also requires GLOII along with GLOI. It is likely that GLOII is also enriched in the epithelium, but this requires verification.

Our finding that GLOI activity decreased in aging human lenses agrees with previous findings (Haik et al. 1994). However, in that study specific activity of the enzyme was not determined and therefore we are not able to compare activities in the two studies. Nevertheless, the present study suggests that the loss of enzyme activity could result in retention of MGO and GO and, consequently, more dicarbonyl reaction products in lens proteins. Several MGO- and GO-derived products, such as MOLD, GOLD, MODIC, argpyrimidine and methyl-hydroimidazolones, have been shown to accumulate with age in the human lens (Biemel et al. 2002; Chellan and Nagaraj 1999; Haik et al. 1994; Padayatti et al. 2001b), probably due to decreased GLOI activity. The loss of GSH in the aging lens (Lou 2000) may further exacerbate the problem, as GSH is required for GLOI activity. The net

effect could be increased protein fluorescence (by argpyrimidine) and protein crosslinking (by GOLD, GODIC, MOLD and MODIC), both of which are characteristics of aging lenses.

Our monoclonal antibody enabled us to measure GLOI immunoreactivity (content) in the human lens. The ELISA measurement suggested a decline of GLOI in the aged lenses. This potentially could be due to proteolytic degradation, as several enzymes have been observed to show decreased activity in aged lenses when compared to young lenses (Jedziniak et al. 1986; Rathbun and Bovis 1986). Such degradation could occur through proteases and the proteosome system present in the lens (David and Shearer 1989; Shang et al. 1997). The loss of immunoreactive enzyme could also be due to masking of the reactive epitope from posttranslational modification processes, such as, glycation and kynurenination.

GLOI is particularly susceptible for modification by NO, and such modification abolishes its activity (de Hemptinne et al. 2007; Mitsumoto et al. 2000). Both NO and H₂O₂ have been implicated in modification of lens proteins during aging (Ornek et al. 2003; Spector 1995). Thus, the observed loss in enzyme activity in the aged lenses could be partly due to chemical modification by NO and H₂O₂.

In summary, using our new monoclonal antibody, we showed that GLOI is mostly present in lens epithelium and its activity and immunoreactivity are both decreased in the aging lens. We propose that the loss in enzyme activity coupled with decreased protein content results in the promotion of MGO-mediated protein modifications in the aging lens.

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Fig. 1.

a Detection of human GLOI by the monoclonal antibody. Western blotting of recombinant GLOI preparations. Purified proteins were separated on SDS-PAGE, electrophoretically transferred to nitrocellulose membrane and probed with GLOI mAb in combination with an anti-mouse HRP-conjugated antibody as described in "Materials and methods". *Mr* molecular weight markers; *Lane 1*, his-GLOI; *Lane 2*, GST-GLOI; *Lane 3*, GST. **b** Optimization of ELISA. Microplate wells were coated with 0.001–4.0 μ g his-GLOI. Optimum his-GLOI concentration was found to be 0.01 μ g that gave an OD of 0.4 within 30 min

Fig. 2.

a Detection of GLOI in human lens and in cultured human lens epithelial cells.

Immunohistochemistry. High immunoreactivity was found in the anterior epithelium for GLOI (experimental, *red*). Nuclear staining was done with DAPI (*blue*). **b** GLOI in cultured human lens epithelial cells. HLE-B3 cells were immunostained for GLOI using GLOI mAb in combination with Texas-Red conjugated anti-mouse antibody (*red*). Cells were counter-stained with DAPI for nucleus (*blue*). For negative control, lens sections and cells were processed identically as above except that the primary antibody was deleted (color figure online)

Fig. 3.

a GLOI activity assay. MGO and GSH (2 mM each) were incubated for 10 min at room temperature to generate hemithioacetal substrate for GLOI. To this, recombinant his-GLOI or human lens water-soluble protein was added and incubated at room temperature and the absorbance due to *S*-lactoylglutathione was monitored at 240 nm. **b** Effect of age on GLOI activity in human lenses. The GLOI activity was measured in the water-soluble fraction of human lenses. Data were submitted to linear regression analysis using StatView 5.0 software (SAS Institute Inc, Cary, NC)

Fig. 4.

Effect of age on the GLOI immunoreactivity in human lenses. Human lens water-soluble protein (2 μ g) was coated onto microplate wells. Immunoreactivity (absorbance at 450 nm) was detected using GLOI monoclonal antibody in combination with a HRP-conjugated anti-mouse antibody as described in "Materials and methods". Data were submitted to linear regression analysis using StatView 5.0 software. Inset shows Western blotting for GLOI in three representative human lenses. Water-soluble proteins from a young (Y 8 years), middle aged (MA 46 years) and aged (A 70 years) were separated on SDS-PAGE, electrophoretically transferred to nitrocellulose membrane and probed with GLOI antibody in combination with a HRP-conjugated anti-mouse secondary antibody. Glyceraldehyde-3-phosphate dehydrogenase (GAP-DH) was used as the loading control