The role of clusterin in retinal development and free radical damage

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EXTENDED REPORT

The role of clusterin in retinal development and free radical damage

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Aim: To assess the role of clusterin in retinal vascular development and in free radical damage in vivo and in vitro.

Methods: The expression of clusterin, von Willebrand factor (vWF), flk-1, heat shock protein 27 (Hsp27) and heat shock protein 70 (Hsp70) was examined in the retinas of developing mice and oxygen-induced retinopathy (OIR) mice by immunofluorescence staining and western blot analysis. Hydrogen peroxide (H_2O_2)-pretreated human retinal endothelial cells (HREC) and astrocytes were cultured in the presence or absence of exogenous clusterin, and then the cell viability was measured using the MTT assay and DAPI staining.

Results: Clusterin was expressed mainly in the inner retina and co-localised with vWF, an endothelial cell marker. During the mouse developmental process, clusterin expression was decreased, which was similar to the expression of flk-1, vWF and Hsp27. Furthermore, in the OIR model, clusterin expression changed in a similar way to both vWF and Hsp27. Under hypoxic conditions, clusterin expression increased in HREC and astrocytes. In H_2O_2-pretreated HREC and astrocytes, clusterin protected against apoptotic cell death.

Conclusions: These results suggest that clusterin is associated with protection from apoptotic retinal cell death in retinal development and in free radical damage.

Clusterin is a glycoprotein first isolated from ram rete testis fluid and has also been termed apolipoprotein J, sulfated glycoprotein-2, glycoprotein III and testosterone repressed message-2.[1–4] It is composed of two 35–40 kDa subunits (α and β) encoded by a single gene and forms a heterodimer stabilised by disulfide bonds. Clusterin has been implicated in diverse physiological functions.[5 6] In animal tissues clusterin mRNA is nearly ubiquitous, being found in tissues as diverse as the rat prostate gland and quail neuroretinal cells. Across species, clusterin maintains a high level of sequence homology (70–80% between mammals).[7] The wide distribution and sequence conservation of clusterin suggest that the protein performs a function of fundamental biological importance. In addition, there is extensive evidence of a correlation between clusterin expression and disease (for example, Alzheimer's disease, gliomas) or pathological stress (for example, ischaemic insult of the kidney).[8–10]

Furthermore, recent studies of clusterin knockout mice have suggested that clusterin protects mice from the pathological consequences of inflammation[11 12] and age-dependent deposition of antibody-containing aggregates in the kidney.[13] Furthermore, the clusterin promoter contains a highly conserved 14-bp element, which is recognised by the transcriptional regulator heat shock factor 1.[14] Heat shock factor 1 activates expression of both clusterin and heat shock proteins, which protect cells from stress.[14] An emerging theme is that clusterin is a protective molecule that is upregulated during times of physiological stress such as neuronal development.[15] However, the function of clusterin in retinal development remains unknown.

In the present study, we investigated the expression of clusterin in the developing mouse retina and in animal models of oxygen-induced retinopathy (OIR) to define the role of clusterin in retinal vascular development. We further investigated the possible protective activity of clusterin, particularly against H_2O_2-induced apoptosis, using in vitro cell cultures.

MATERIALS AND METHODS

Animals

C57BL/6 mice were purchased from Samtako (Korea). Care, use and treatment of all animals in this study was in strict agreement with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research. C57BL/6 mice were kept in standard 12-hour dark-light cycles and approximately 23℃ room temperature. Eyes for normal retina development experiments were enucleated from C57BL/6 mice sacrificed postnatally on days P6, P8, P10, P12, P14, P16 or from adult mice (P21, P26). Each group contained 10 animals.

Oxygen-induced retinopathy

Oxygen-induced retinopathy was induced as described by Zhang *et al*[16] with some modifications. Briefly, newborn mice were randomly assigned to experimental and control groups. At postnatal day 7 (P7), mice in the experimental group were exposed to hyperoxia (80% O_2) for five days (P7 to P12) and then returned to normoxia (room air). Control mice were kept at constant normoxia. Eyes were enucleated from mice sacrificed on P6, P10, P12, P14, P16, or P21.

Mice retinal tissue preparation

After cervical dislocation the eyes were enucleated and hemisected at the ora serata. The retinas were gently teased off the sclera using a fine brush. Contamination by RPE cells was reduced to a minimum. Whole retinal proteins were extracted with lysis buffer (50 mM Tris (pH 7.6), 150 mM NaCl, 1% Triton X-100, 0.1% SDS, protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO, USA), 1 mM PMSF) on ice for 20 min, centrifuged at 14 000 × rpm for 20 min, and then supernatants were harvested and stored at −80℃.

Abbreviations: GCL, ganglion cell layer; HREC, human retinal endothelial cells; Hsp, heat shock protein; INL, inner nuclear layer; IPL, inner plexiform layer; OIR, oxygen-induced retinopathy; vWF, von Willebrand factor

Immunohistochemistry

The enucleated mouse eyes used for imunohistochemistry were immersion fixed in 4% formalin and subsequently embedded in paraffin. 4 μm-thick serial sections were prepared from paraffin blocks. Sections were deparaffinised and hydrated by sequential immersion in xylene and graded alcohol solutions, treated with proteinase K for 5 min at 37℃ and then treated with normal serum obtained from the same species in which the secondary antibody was developed for 10 min to block non-specific staining. Slides were incubated overnight at 4℃ with rabbit polyclonal antibody against clusterin (1:100, Santa Cruz, CA, USA) or vWF (1:100, Chemicon, MA, USA). FITC-conjugated IgG (1:400) and TRITC-conjugated IgG (1:400, Jackson ImmunoResearch, Philadelphia, PA, USA) were used as secondary antibodies. The slides were mounted Faramount Aqueous mounting medium (DAKO, CA, USA) and observed under fluorescence microscope.

Cell culture

Human retinal endothelial cells (HRECs) were purchased from Applied Cell Biology Research Institute (ACBRI, USA) and cultured in M199 (Gibco BRL, Invitrogen, Carlsbad, CA, USA) supplemented with 20% FBS (Gibco BRL), 3 ng/ml bFGF (Upstate Biotechnology, Charlottesville, VA, USA), 10 U/ml heparin (Sigma), and antibiotics (Gibco BRL). HRECs were cultured in plates coated with 0.3% gelatin (Sigma-Aldrich). CTX-TNA2, a rat astrocyte cell line, was purchased from the America Type Culture Collection (ATCC) and cultured in DMEM (Gibco BRL) supplemented with 10% FBS and antibiotics. For the experiments, cells were grown to 90–95% confluence.

Purification of clusterin

Clusterin was purified from fresh normal human plasma as previously described.[17] Human plasma was precipitated using 12–23% polyethylene glycol (molecular weight 3350, Sigma) overnight at 4℃. This precipitate was dissolved and subjected to diethylamino ethanol-sepharose and heparin-sepharose column chromatography (GE Healthcare Life Sciences, Piscataway, NJ, USA). Clusterin-positive fractions were then applied to a clusterin monoclonal antibody affinity chromatography column. The anti-clusterin monoclonal antibody (1G8) was generated using recombinant human full-length clusterin expressed in *Escherichia coli* as an antigen, and covalently conjugated to Cyanogen Bromide-Activated Sepharose 4B (Sigma). The eluted protein was dialysed against PBS and stored at −80℃.

Preparation of cell lysates and media

Media samples were harvested and concentrated by freeze-drying. Cells were washed with PBS (pH 7.3) and lysed using lysis buffer, described above, on ice for 30 min, and then centrifuged at 14 000 × rpm for 30 min. Supernatants were stored at −80℃.

Western blotting analysis

Western blotting was performed using standard western blotting methods. The protein concentration in the cytosolic fraction was measured using a BCA protein assay kit (Pierce, Rockford, IL, USA). Anti-flk-1, anti-clusterin and anti-Hsp70 antibodies were purchased from Santa-Cruz Biotechnology and anti-Hsp27 antibody was purchased from Abcam (Cambridge, UK). For western blot analysis, anti-flk-1, anti-clusterin, anti-Hsp70, anti-Hsp27 and anti-β-actin antibodies were used at the concentration of 1:1000, and horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG were used at 1:5000 dilution. To ensure the equal loading of protein in each lane, the blots were stripped and reprobed with an antibody against β-actin. Intensity values were normalised relative to control values. The blots were scanned using a flatbed scanner and the band intensity analysed using the TINA software program (Raytest, Staubenhardt, Germany).

Cell viability assays

Cell viability was evaluated with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. 2×10^4 cells were plated in 96-well plates and cultured overnight. Cells were treated with exogenous clusterin (1 μg/ml) with or without 50 μM H_2O_2 for 2 h. The medium was then replaced with fresh medium containing 0.5 mg/ml MTT for 4 h. After incubation, the medium was carefully removed from the plate and DMSO was added to solubilise formazan produced from MTT by the viable cells. Absorbance was measured at 540 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

DAPI staining

Sterilised round slide cover glasses were placed in 12-well plates, and cells were plated in the wells. Cells were treated with exogenous clusterin (1 μg/ml) with or without 50 μM H_2O_2 for 2 h. Cells were washed twice with PBS (pH 7.3) and fixed in 1% paraformaldehyde for 10 min at room temperature. After fixation, cells were washed three times with washing buffer (0.1% Triton X-100 in PBS, pH 7.3) and treated with 10 μg/ml DAPI (Sigma). After incubation for 5 min in the dark, the cells were washed three times with washing buffer. The cover glasses were removed and mounted on slides. The stained cells were visualised by fluorescence microscopy.

Statistical analysis

Data are expressed as mean (standard deviation). Comparisons between controls and treated groups were performed using the Student t test. A p value <0.05 was considered statistically significant.

RESULTS

Localisation of clusterin in the developing mouse retina

We examined the expression and the localisation of clusterin in the developing retina using immunofluoresence staining. As shown in figure 1, at P8, immunolabelling of clusterin was detected in the ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), and outer plexiform layer. At P16, there was strong immunoreactivity in the inner retina including the GCL, IPL and INL and, at maturity (P26), clusterin was expressed in the inner retina including the retinal vessels. Blood vessel development was characterised by immunostaining of vWF. Double staining of clusterin and vWF show that clusterin was colocalised with vWF, which shows clusterin was expressed in the endothelial cells. In the negative control staining, no positive immunoreactivity was found in any layers of the retina (data not shown).

In mammals, retinal blood vessels are first evident in the inner retina surrounding the optic disc and later grow towards the peripheral retinal margin.[18 19] Thus, clusterin expression in the inner retina and co-localisation with vWF might suggest a correlation between clusterin and retinal blood vessel development.

Expression patterns of clusterin in retinal development and in OIR model

To access the changes in clusterin expression during mice retinal development, we used western blotting to examine developing retina tissues from P6 to P21 mice. As shown in figure 2, clusterin expression decreased during mouse retinal development, in a pattern similar to flk-1 expression, which is

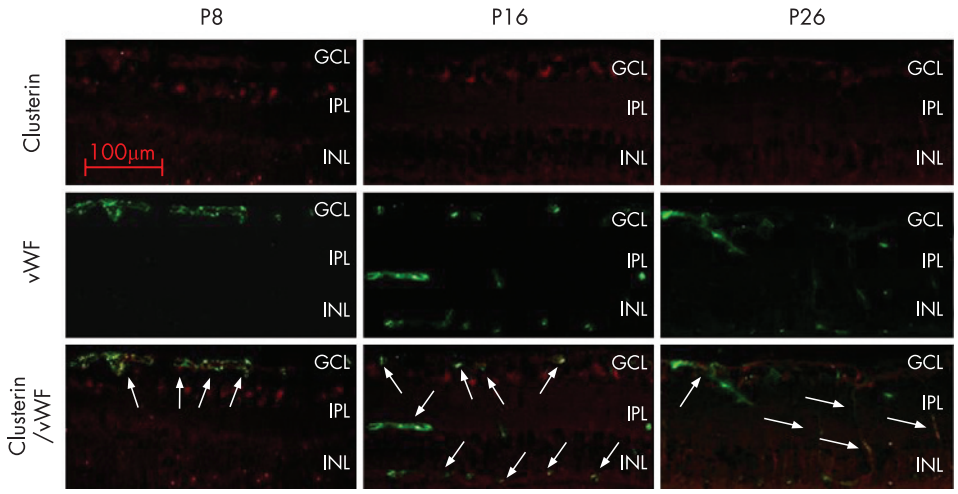

Figure 1 Immunofluorescence staining of clusterin and vWF in the developing mouse retina. Sections of retina from mice of varying ages (P8, P16, P26) were immunostained with antibodies against clusterin (red) and Hsp27 (green). GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer. Arrows indicate the co-localisation of clusterin with vWF (magnification ×200).

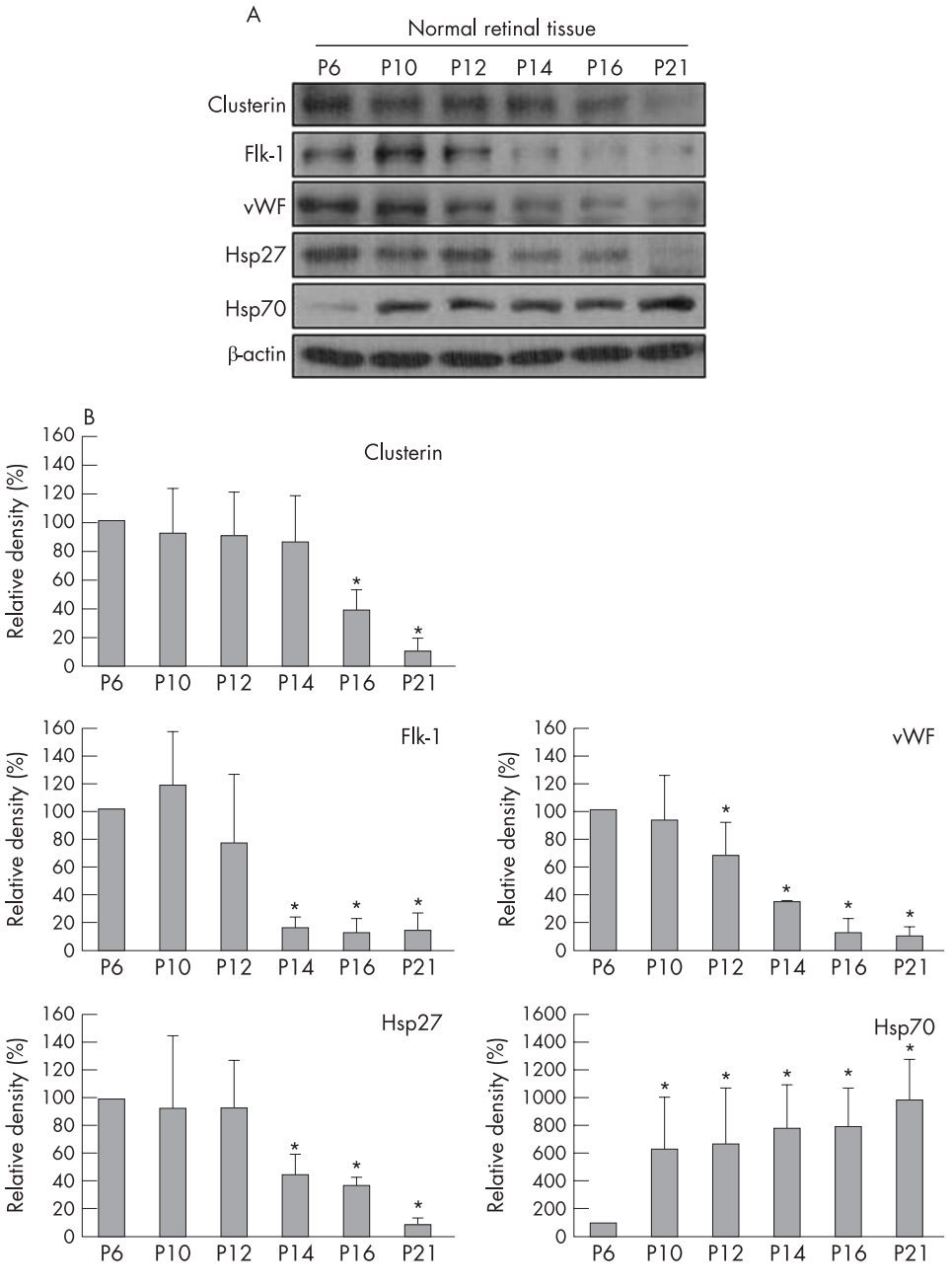

Figure 2 Expression patterns of flk-1, vWF, clusterin, Hsp27 and Hsp70 during mouse retinal development. (A) Retinal proteins from P6–P21 mice were analysed by SDS-PAGE and western blot analysis. β-actin served as a loading control. (B) Quantitative analysis was performed by measuring protein expression relative to the control. Each point represents the mean (SD) of three independent experiments, each performed in triplicate. *$p<0.005$.

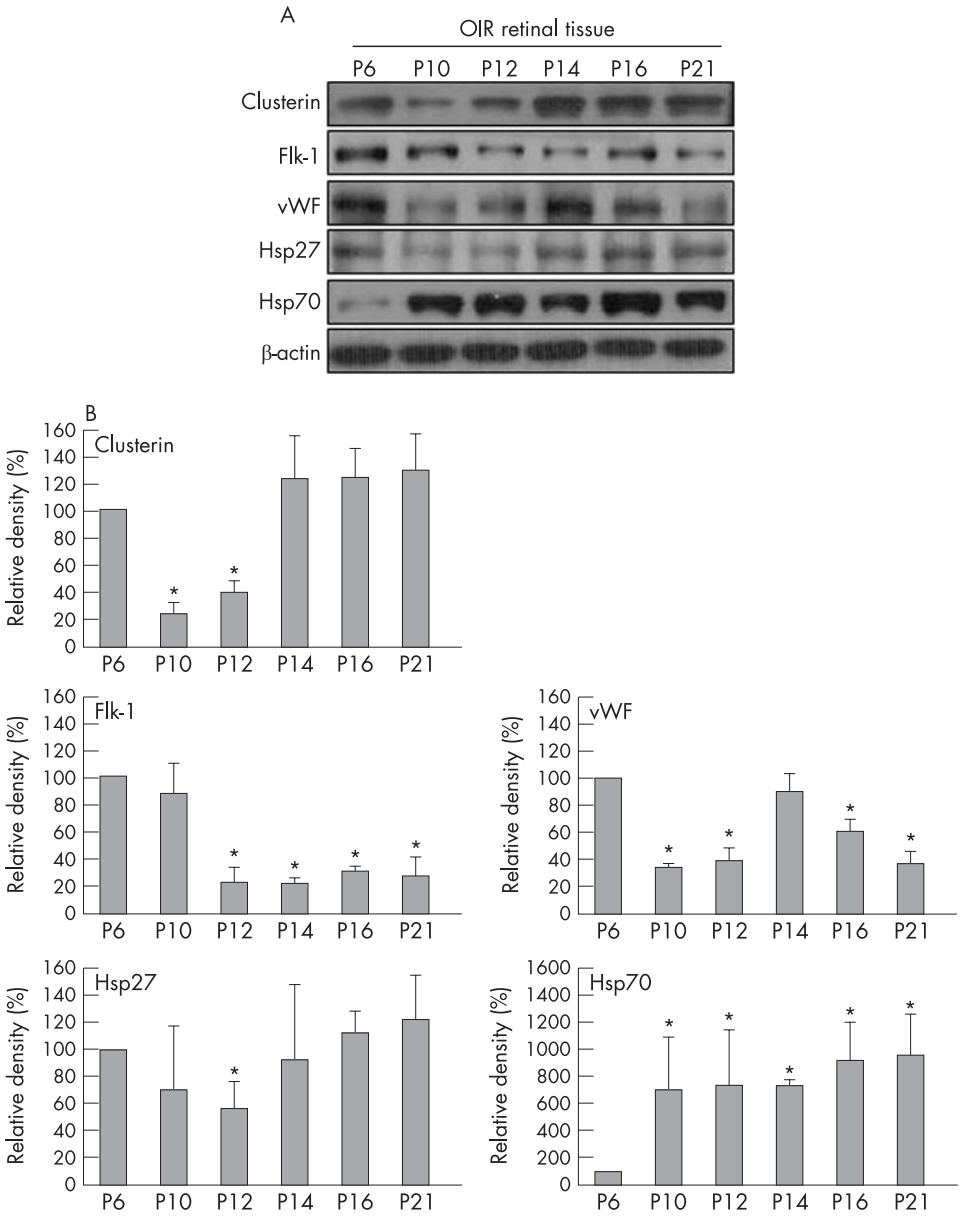

Figure 3 Expression patterns of flk-1, vWF, clusterin, Hsp27 and Hsp70 in retinas of oxygen-induced retinopathy (OIR) mice. At P7, mice were exposed to hyperoxia (80% O_2) for five days (P7–P12) and then returned to normoxia. (A) Retinal proteins of OIR mice retina from P6–P21 were analysed by SDS-PAGE and western blot analysis. β-actin served as the loading control. (B) Quantitative analysis was performed by measuring protein expression relative to the control. Each point represents the mean (SD) of three independent experiments, each performed in triplicate. *$p<0.005$.

known to play a central role in endothelial cell proliferation and differentiation[20] and vWF, an endothelial cell marker. Hsp27 expression peaked between P6 and P12 and decreased at later times (P14–P21), as reported previously,[21] while Hsp70 expression increased during later times (P14–P21).

In the OIR retina, vWF expression was markedly decreased from P10 to P12, when vascular development was halted, and began to increase at P14, when endothelial cell proliferation is reinitiated (fig 3). Therefore, the vasculature of OIR retina was highly constricted and very few vWF-positive blood vessels remained compared to the normal retina (fig 2) at P10–P12. Clusterin and Hsp27 expression also changed similarly to vWF expression (fig 3). However, Hsp70 expression was not changed in the OIR model. These results indicated that clusterin could play a role in retinal blood vessel development such as small heat shock proteins (sHsps), the molecular chaperones.

Clusterin expression in hypoxic conditions

Development of the retinal vasculature is controlled by a hierarchy of interactions among retinal neurons, astrocytes and blood vessels. Hypoxia caused by the increasing oxidative demands of developing retinal neurons has long been considered to be a driving force in initiation of retinal vessel development.[22 23] Therefore, we examined the effect of hypoxia on clusterin expression in HREC and astrocytes, the major components of retinal vasculature. When HREC and astrocytes were exposed to hypoxic conditions ($O_2<1\%$) for 90 min, clusterin expression was increased both in the cell culture medium and in cell lysates (fig 4). The increase in clusterin was also induced by exposure to hypoxia mimetics such as desferrioxamine mesylate or $CoCl_2$ (data not shown). These results confirmed that clusterin was upregulated under hypoxic conditions such as retinal vessel development in both HREC and astrocytes.

Clusterin protects against apoptotic cell death

To investigate the role of clusterin in retinal vessel development, we used purified clusterin as described in the Materials and methods section. HREC and astrocytes were treated with purified clusterin (1 μg/ml) with or without 50 μM H_2O_2 for 2 h then cell viability was measured using the MTT assay. Exposure of HREC or astrocytes to H_2O_2 induced apoptotic cell

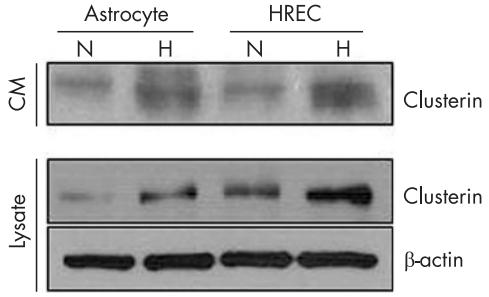

Figure 4 Increase of clusterin expression in hypoxic conditions. Human retinal endothelial cells (HREC) and astrocytes were exposed to normoxic (N) or hypoxic (H) condition (O_2<1%) for 90 min, and then cell culture media or cell lysates were assessed by western blot using an anti-clusterin antibody. β-actin served as a loading control.

death, while cells treated with clusterin (1 μg/ml) were protected from H_2O_2-induced apoptosis (fig 5A). These results indicate a protective effect of clusterin in both HREC and astrocytes. This protective effect of clusterin was also detected by DAPI staining to visualise the morphological changes in chromatin (fig 5B). No fragmented DNA was detected in normal HREC or astrocytes, while many strong fluorescent spots, indicating apoptotic bodies, were detected by DAPI staining of H_2O_2-treated cells. However, when the cells were co-treated with H_2O_2 and clusterin, the amounts of apoptotic cells were significantly reduced. These results indicate that clusterin could protect the HREC and astrocytes from apoptotic cell death.

DISCUSSION

In mouse retinal vasculature development, the primary vascular network spreads approximately halfway across the inner surface of the retina by P4 and reaches the periphery approximately 1 week (P7) after birth. After the vascular network has spread across the entire retina, arteries and veins strictly alternate and, at approximately P7, start to sprout downwards, into the inner plexiform layer, where they establish a second vascular network parallel to the first by P27.[24–26] It is widely held that the primary vascular development across the inner surface of the retina occurs by vasculogenesis, whereas the establishment of the secondary network in the inner plexiform layer occurs by angiogenesis.

We have investigated the expression and cellular localisation of clusterin in the retinas of normally developing mice and in those of OIR mice. OIR is a model for human retinopathy of prematurity (ROP), a major cause of blindness in children. Using western blotting and immunohistochemistry, we confirmed that clusterin expression accompanies retinal vessel development, which is detected by flk-1 and vWF expression. Interestingly, in the OIR retina, clusterin expression was markedly decreased from P10 to P12, when vascular development was interrupted. These results indicate that clusterin expression might be related to retinal vessel development.

The Hsp27 immunoreactivity in the developing retina coincided with that of clusterin, as both were detected mainly in the GCL, in which large vessels and extensive capillary beds are found (data not shown). Hsp27, a member of the sHsps family, functions as a molecular chaperone in both neuronal and non-neuronal cells.[27] Recent studies reported that Hsp27 overexpressed in human endothelial cells during post-hypoxic reoxygenation can protect cells from delayed apoptosis.[28] Furthermore, Hsp27 upregulation by hypoxia-inducible factor-1 signalling offers protection against retinal ischaemia in rats.[29] Thus, the similarities in localisation and expression patterns between clusterin and Hsp27 support our hypothesis that clusterin also plays a protective role during retinal vessel development.

There is abundant evidence that clusterin expression is increased during cellular stresses. Previous studies have reported that overexpression of clusterin protects cells from TNF-α[30 31] and that clusterin anti-sense transfectants have reduced cellular resistance to TNF-α,[31] heat shock and oxidative stress.[32]

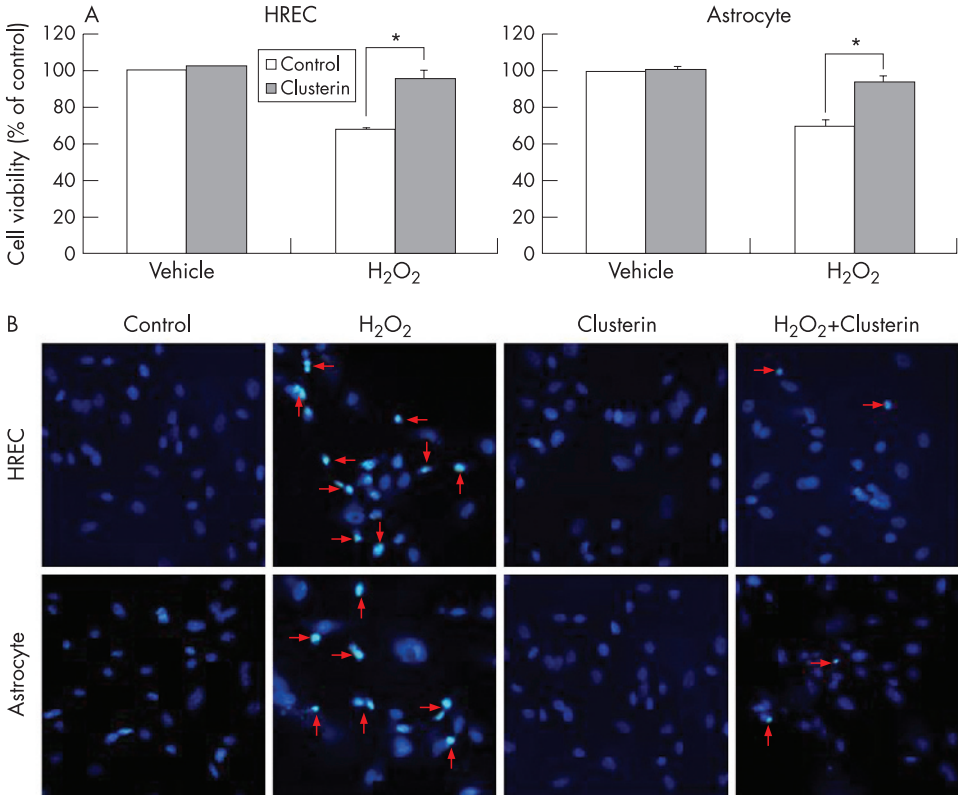

Figure 5 Protective effects of clusterin on human retinal endothelial cells (HREC) apoptosis. (A) HREC and astrocytes were treated with purified clusterin (1 μg/ml) with or without 50 μM H_2O_2 for 2 h and then cell viability was measured using the MTT assay. Data are shown as per cent change from control. Each point represents the mean (SD) of three independent experiments, each performed in triplicate. *p<0.005. (B) HREC (a–d) and astrocytes (e–h) were treated with purified clusterin (1 μg/ml) with or without H_2O_2 for 2 h and then stained with DAPI. (a, e) Control cells. (b, f) Cells treated with 50 μM H_2O_2. (c, g) Cells treated with clusterin. (d, h) Cells treated with clusterin and 50 μM H_2O_2. Arrows indicate apoptotic cell nuclei. (×200).

Furthermore, purified clusterin added to cell culture medium protects the cells from TNF-α[33] and from oxidative stress.[34]

Thus, multiple lines of evidence indicate that clusterin expression is increased during cellular stresses and that clusterin exerts cytoprotective effects.

The chaperone action of clusterin could be cytoprotective in either or both the intra- or extracellular environments. Recent studies have suggested that sHSPs and clusterin are molecular chaperones that share many functional similarities despite their lack of significant sequence similarity.[35] sHsps are ubiquitous intracellular proteins whereas clusterin is generally found extracellularly. Both sHsps and clusterin have little ability to refold target proteins and their chaperone action does not require ATP hydrolysis. Increased expression of sHsps and clusterin accompanies a range of diseases that arise from protein misfolding and deposition of highly structured protein aggregates known as amyloid fibrils, for example in Alzheimer, Creutzfeldt-Jakob and Parkinson diseases.[36]

A recent study indicated that clusterin may play a role in modulating the vascular smooth muscle cell (VSMC) response to injury, in part by inhibiting cell migration, adhesion and proliferation.[37] Clusterin also inhibits endothelial cell migration and adhesion by altering endothelial function during vascular injury. In this latter study, clusterin appears to regulate the early development of intimal hyperplasia after prosthetic arterial grafting.[38] In our experiments, exogenous clusterin-treated cells were protected from H_2O_2-induced apoptotic cell death and this protective effect of clusterin was detected in both astrocytes and HREC, which are important in retinal vasculature.

In conclusion, clusterin shows a protective effect from apoptotic retinal cell death in retinal development and free radical damage. In vitro, clusterin potently inhibits stress-induced cell death and, in vivo, clusterin expression appears to be associated with retinal vessel development. Our data indicate that clusterin expression is upregulated in cells undergoing hypoxia, which is considered to be a driving force in initiation of retinal vessel development. Furthermore, clusterin protects cells from stress and promotes cell viability. On the basis of the available evidence, we propose that clusterin may act to protect cells from stress during retinal vessel development.

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