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Vitreous levels of oxidative stress biomarkers and the radical-scavenger α_1 -microglobulin/A1M in human rhegmatogenous retinal detachment

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Keywords: oxidative stress, retinal detachment, vitreous, antioxidant, alpha-1-microglobulin.

ABSTRACT

Purpose. To explore oxidative stress and the radical scavenger α_1 -microglobulin (A1M) in the vitreous body of human eyes with primary rhegmatogenous retinal detachment (RRD).

Methods. Levels of carbonyl groups, a marker of oxidative stress, and A1M were measured by ELISA and RIA in 14 vitreous samples derived from patients suffering from RRD and compared with 14 samples from macula hole (MH) patients. Carbonyl group and A1M levels in RRD samples were statistically related to detachment characteristics. Analysis of total protein level, SDS-PAGE, and Western blotting of A1M was also performed. In a separate experiment, mRNA expression of A1M was measured by RT-PCR in rat retina explants.

Results. Levels of carbonyl groups and A1M varied widely in RRD vitreous samples but was significantly higher in samples derived from eyes with large detachment area and macula-off status while the presence of vitreous hemorrhage did not show any significant correlation. Compared with MH samples, RRD samples displayed significantly higher levels of A1M, whereas changes in total protein levels and carbonyl groups were not significant. Novel forms of A1M, not previously seen in plasma, were found in the vitreous body by Western blotting. Furthermore, A1M expression was seen in rat retina explants and was upregulated after 24h of culturing.

Conclusion. Oxidative stress is a prominent feature of human eyes with primary RRD, and is directly related to detachment severity. Affected eyes can launch a protective response in the form of the radical scavenger A1M possibly derived from the retina. The results thus indicate potential therapeutic cell loss prevention in RRD by employing the endogenous radical scavenger A1M.

INTRODUCTION

The term "oxidative stress" is used to describe conditions with an abnormally high production of redox active compounds and/or impaired antioxidative tissue defence systems [1]. Major mediators of oxidative stress are reactive oxygen species (ROS) including free radicals, which are extremely reactive compounds due to the presence of unpaired electrons. ROS include hydrogen peroxide (H_2O_2) and the hydroxyl and superoxide radicals, which induce oxidative stress by oxidative reactions with cellular and extracellular molecular components. One of the most important generators of ROS is free hemoglobin (Hb), released from red blood cells during haemorrhage and hemolytic conditions [2].

Normally, ROS and other oxidants are counteracted by antioxidants including the high-molecular weight enzymes superoxide dismutase (SOD), catalase, and glutathione peroxidases and the low-molecular weight non-enzymatic compounds glutathione, vitamin C and E. These compounds have previously been well characterized, but novel antioxidants are continuously being discovered. One such molecule is the radical scavenger A1M (α_1 -microglobulin) which was recently shown to have protective properties against oxidative stress in cell cultures, skin and placenta [3-5] (reviewed in [6]). A1M is a ubiquitous low molecular weight (26 kDa) plasma and tissue protein [7, 8] mainly synthesized in the liver [9, 10] but also, in less amounts, in peripheral organs such as blood cells, pancreas and kidney. From the liver cells, A1M is secreted to the blood stream where it is found in a free form ($\sim 1 \mu M$) and as high-molecular weight complexes with IgA, albumin and prothrombin ($\sim 1 \mu M$) [11, 12]. It is rapidly distributed to several tissues where it is transported from the blood vessels to the extravascular compartments [13]. A1M is a reductase, [14] a multispecific scavenger of small organic radicals [15] and has

antioxidant properties [3]. In addition, an increased synthesis in liver, blood cells, placenta and skin keratinocytes is induced by cell-free Hb and ROS [4, 5, 16].

The eye is continuously subjected to oxidative stress from both exogenous and endogenous sources, and impaired redox balance has been implicated in a number of ophthalmologic disorders, *e.g.* cataract of the lens [17-19], diabetic retinopathy [20-22], and age-related macular degeneration (AMD) [23-25]. Recent findings indicate that oxidative stress may also play a role in experimental retinal detachment (RD), and that ROS scavenger treatment can attenuate RD-related photoreceptor death [26, 27, 28]. Given the potential clinical importance, in this paper we have explored the presence of biomarkers of oxidative stress, as well as properties of the protective molecule A1M in human eyes with primary RRD.

MATERIALS AND METHODS

Patients

Vitreous samples were obtained from 14 phakic eyes of 14 patients during vitrectomy for primary RRD at the University Hospital of Lund. RRD after previous vitreoretinal surgery and secondary to trauma was not considered primary, and such samples were thus not included as well as samples from eyes with concomitant eye disease including cataract. Fourteen cases of macular hole (MH) were included for comparison resulting in a total of 28 samples. Written consent was obtained from each patient, and all procedures complied with the Declaration of Helsinki.

Approximately 1.0 ml of undiluted vitreous was obtained from each eye under air infusion at the initial stage of each procedure. Vitreous samples were immediately refrigerated at -20° C, and after 1 hour frozen at -80° C. Before analysis, the samples were thawed, weighed, and Complete Mini Protease inhibitor (Roche Diagnostics, Germany) was added to 5% (w/w).

Background data for a number of preoperative variables for RRD patients were obtained, as well as characterization of the detachments including presence of vitreous hemorrhage, extent of detachment and macular status (Table 1). Detachment characteristics were grouped and statistically analyzed in relation to vitreous concentrations of carbonyl groups and A1M using two-tailed Student's t-test (see below, Fig. 2). GraphPad InStat, GraphPad Software, San Diego California USA, was used for all calculations. Statistical significance was defined as $p < 0.05$ using the above-mentioned tests.

Total protein analysis and SDS-PAGE

Total protein in the vitreous samples was measured in a Bradford assay as described by Bradford [29]. To investigate the protein contents of the vitreous samples, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli [30], using gels bought from Thermo Scientific containing 12% or 4-20% polyacrylamide. Reduced conditions were achieved by mixing the samples with sample buffer containing 2% (v/v) mercaptoethanol and then boiling for 1 minute before applying them to the gel. Samples were centrifuged prior to analysis. Electrophoresis was performed at 150 V for about 45 minutes. Gels were then stained with Coomassie Brilliant Blue R-250 (BDH Chemicals, Ltd. Poole, UK) and dried.

Measurement of A1M concentrations

Radioimmunoassay (RIA) was performed as described previously [31] to measure the concentration of A1M in the vitreous body samples. Briefly, polyclonal goat anti-A1M (HALVAN, prepared at our laboratory as described [32]), diluted 6000 x, was mixed with ¹²⁵I-human urine A1M, approximately 50 ng/ml, and standard A1M or unknown samples, and incubated over-night at RT. Bovine serum and polyethylene glycol 6000 were then added to 20% and 10%, respectively, the samples centrifuged and the pellets analyzed in a Wallac Wizard 1470 gamma counter (Perkin–Elmer Life Sciences). ¹²⁵I-labeling of A1M was done using the chloramine T method [33]. Protein-bound iodine was separated from free iodide by gel-chromatography on a Sephadex G-25 column (PD10, Amersham-Pharmacia Biotech). A specific activity of around 0.1 MBq/μg protein was obtained.

Western blotting of A1M

The different forms of A1M in the vitreous samples were analyzed by Western blotting, performed as described previously [34]. Electrophoresis was performed as described

above. Instead of staining, the proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Immobilon, Millipore, Bedford, MA) as described [35]. Polyclonal rabbit anti-human A1M (K:107, prepared at our laboratory as described [36]), diluted 2000 x, was used as primary antibody, incubated over-night at 4°C. As secondary antibody ¹²⁵I-goat anti-rabbit IgG [32], incubated 1 h at RT, was used.

Carbonyl group ELISA

Measurement of oxidative stress was performed using a carbonyl group ELISA essentially as described [37]. Vitreous body sample was diluted to 0.17 mg total protein/ml and 12.5 µl were then derivatized with DNP-hydrazine (Sigma Cat nr. D-2630). Anti-DNP (Invitrogen), diluted 2000x, was used as primary antibody, incubated 3h at RT. Swine-anti-rabbit IgG (Dako A/S), diluted 2000x, was used as secondary antibody, incubated 1h at RT. O-phenylenediamine (Sigma Cat nr. P5412), diluted in 60mM Tris-HCl, pH 8.5, was used as substrate solution and absorbance was measured at 450nm.

Expression of A1M-gene in retina explants

To quantify expression of the A1M-gene in retinal cells, rat retinas were removed from five-month old Sprague-Dawley rats (18 eyes). The rats were killed with CO and then decapitated. The eyes were removed and the neuroretinas carefully dissected free from the retinal pigment epithelium (RPE) with fine forceps. The optic nerve was thereafter cut with microscissors, and the neuroretina washed twice in phosphate buffered saline (PBS, 10 mM Na-phosphate pH 7.4, 125 mM NaCl) and incubated at 37°C for 3h in PBS, 200 µl for each eye. The medium was aspirated, the tissue solubilized in 1 ml Trizol (Invitrogen, cat nr. 15596-018), and then stored in -80°C until used for real time-PCR analysis. Messenger RNA was isolated from the Trizol-solubilized retina tissue, prepared as described above. Reverse Transcription PCR reagents (Fermenta) were used to transcribe mRNA to cDNA.

Real time-PCR was then performed using the following primers, for A1M:

TTCTTGTTGCTGACTGCCTGCC (forward), TTCTTAATCCGCCTCAGCCACG

(reverse) and for the housekeeping gene glyceraldehyde-3-phosphate-dehydrogenase

(GAPDH) TGAACGGGAAGCTCACT (forward), TCCACCACCCTGTTGCTG

(reverse). The primers were obtained from Eurofins MWG Operon. The expression was

analyzed using iQ SYBR Green Supermix (Bio-Rad). Raw data were obtained as cycle

threshold values (Ct-values) and were normalized to the Ct-values of human GAPDH.

Alternatively, rat full-thickness neuroretinas (n=10) were explanted on culture plate inserts

(Millicell-HA 0.45- μ m; Millipore, Billerica, ME) with the photoreceptor layer toward the

membrane. The explants were cultured in 2 mL Dulbecco's modified Eagle's medium

(DMEM)/F12 medium-L-glutamine (Gibco) supplemented with 10% fetal calf serum. A

cocktail containing 2 mM L-glutamine, 100 U/mL penicillin, and 100 ng/mL streptomycin

(Sigma-Aldrich, St Louis, MO) was added, and the retinas were maintained at 37°C with

95% humidity and 5% CO₂. Specimens were kept under culture conditions for 24 hours,

and A1M was thereafter analysed by real time-PCR as described above.

All proceedings and animal treatment were in accordance with the guidelines and

requirements of the Government Committee on Animal Experimentation at Lund

University and the "Principles of laboratory animal care" (NIH publication No. 85-23,

revised 1985), the OPRR Public Health Service Policy on the Humane Care and Use of

Laboratory Animals (revised 1986) and the U.S. Animal Welfare Act, as amended, were

followed.

RESULTS

Total protein analysis

Total protein was measured in a Bradford assay (Table 2). The mean amount of total vitreous protein in RRD samples was 0.78 mg/ml \pm 0.18 SEM and in the MH samples 0.52 mg/ml \pm 0.18 SEM. This difference was not significant ($p=0.32$ two-tailed Student's t-test). The SDS-PAGE (Fig. 1) showed that the 66kDa albumin band was present in all samples. IgG light chain could be seen as a band at approximately 25kDa in sample R3, R5, R6 and R12. A band at 17kDa was also seen in some samples, corresponding to single hemoglobin chains by comparing their migration to exogenously added hemoglobin chains (not shown). The amount of hemoglobin in the different samples was estimated by visual analysis of the SDS-PAGE and the results are presented in Table 2.

Carbonyl groups

Oxidative stress was measured by the presence of protein carbonyl groups, which are produced as a result of oxidation reactions in the vitreous. The concentrations of carbonyl groups in each sample, expressed as absorbance units/ μ l sample, are shown in Table 2. Since the total volume of all samples was approximately the same (around 1 ml, see Materials and Methods), these values are thus proportional to the total amount of oxidative stress exerted in the vitreous. In the RRD group, the mean values of carbonyl groups measured 0.087 (abs units/ μ l) \pm 0.021 SEM, the macular hole samples 0.062 \pm 0.014 SEM. The increased amount of carbonyl groups in the RD samples was not statistically significant ($p<0.34$; see Fig 2).

Concentrations of A1M

The mean concentration of A1M in retinal detachment samples was $0.50 \mu\text{g/ml} \pm 0.12$ SEM, in the macular hole samples $0.21 \mu\text{g/ml} \pm 0.061$ SEM (Table 2). The A1M concentration in the RRD samples was significantly higher compared to the MH control group ($p=0.041$; see Fig. 2). To account for a possible leakage of plasma as a source of A1M in the vitreous samples, the A1M-concentrations were divided by the total protein concentrations and compared to a plasma sample (not shown). In patients with RRD, the levels of A1M in the vitreous body relative to the total protein content varied widely between the samples, and no apparent correlation between RRD samples and plasma was found.

Correlations with clinical parameters

Since the concentrations of carbonyl groups and A1M in the vitreous may be employed as markers of total oxidative stress and antioxidant capacity, respectively, we separately analysed the correlation of these values with extent of detachment and macula status (Fig. 2). The vitreous concentrations of A1M and carbonyl groups were found to be significantly higher in eyes with extensive detachment and with macula off (Fig. 2). To explore the blood-derived contribution of A1M and carbonyl groups, a separate analysis of samples derived from eyes with or without visible vitreous hemorrhage. The presence of vitreous hemorrhage did not correlate with carbonyl group or A1M concentrations.

Western blotting of A1M

In agreement with previous reports [12], samples of normal human blood plasma displayed an A1M-band at 33kDa representing free 26kDa A1M, a band at 90kDa representing the IgA-A1M complex, a band at 100kDa and 135kDa representing two forms of A1M-albumin, a band at 110kDa representing A1M-prothrombin, and a band at >225 kDa

representing a multimeric form of IgA-A1M. In addition, a band was seen at 20 kDa, representing an A1M-fragment (unpublished data).

In the vitreous from patients with RRD, bands at corresponding molecular weights were found (Fig. 3). However, the intensity of the anti A1M-stained bands varied among samples, which was also the case for the distribution of the various forms. The sample from the patient with macula hole displayed weak bands. In addition, several unique bands in most samples of vitreous body, not seen in plasma, were found. The two most prominent were seen at 66 and 76 kDa.

A1M mRNA expression in rat retina

A1M expression was investigated in non-cultured and cultured rat retinas. To explore A1M expression in the non-cultured normal retina tissue, neuroretinas were kept for 3h in PBS prior to mRNA analysis. We also wanted to investigate the capacity of the retina to upregulate A1M under stress-induced conditions by culturing neuroretinas for 24h. The expression of selected genes were analyzed by real time PCR. A1M mRNA was found both in non-cultured and cultured tissue (Table 3). To compare levels under the different conditions, A1M mRNA levels were normalized to the levels of a house-keeping gene, GAPDH. Relative to GAPDH, the A1M mRNA was more abundant in cultured than non-cultured retina ($\Delta\Delta C_t = 2.27$, Table 3), a statistically significant upregulation ($p < 0.02 * 10^{-5}$).

DISCUSSION

In this paper we have shown that oxidative stress as well as antioxidation are prominent features of human eyes with primary RRD. Macular-off status as well as large detachment extension correlated significantly with the magnitude of carbonyl group manifestation, but also with A1M upregulation. This indicates that oxidative stress is pronounced and proportional to detachment severity, but also that the retina is able to launch a protective response to the injury. Oxidative stress has recently been implicated in experimental RD related cell-death in rodents [26, 27, 28], and we can now confirm that it is relevant also in the clinical situation.

A correlation between A1M-concentrations and oxidative stress markers (carbonyl groups) *in vivo* has been shown previously in pregnant women with preeclampsia [38]. Oxidative stress-induced A1M upregulation has also been shown in several tissues including liver, blood cell lines [16], keratinocyte primary cultures, and skin explants [4]. In the eye, A1M upregulation could theoretically be derived from a disturbed blood-ocular barrier function and/or local upregulation in eye tissues. Previously, substantial A1M production has been reported in the liver from which distribution takes place via the circulation throughout the body [13]. Retinal detachment is associated with blood-ocular barrier breakdown, and it is therefore plausible that A1M in the eye may also be derived from this source. However, we found no apparent correlation between the A1M/total protein quotient in vitreous and plasma samples. Similarly, RRD eyes with vitreous hemorrhage did not show a higher A1M or carbonyl group level. Interestingly, rat retina *in vitro* showed a capacity to upregulate A1M mRNA expression *in vitro*. Put together, the results suggest that at least part of the A1M found in the vitreous is indeed produced by the retina in response to RRD induced oxidative stress.

Further support for the concept of local A1M production can be gained when vitreous isoforms of A1M are examined. High molecular weight (>100 kDa) forms of A1M (*i.e.* complexes with IgA, albumin and prothrombin) have been isolated and characterized in human plasma [12]. These forms were absent in most of our vitreous samples, again indicating a discrepancy between plasma and vitreous. We found that the anti-A1M antibodies consistently stained three novel bands in the vitreous samples, but not in plasma. These bands migrated as 75, 66 and 55 kDa and may represent new, vitreous-specific forms of A1M. An alternative explanation is that these three bands are plasma proteins present in the vitreous recognized by anti-A1M antibodies. The antibodies were produced by immunizing with urinary A1M and are therefore expected to bind to the brown chromophores found on urinary A1M [39, 40]. It was suggested that these represent degradation products of small organic radicals covalently linked to side-chains on A1M, for example the tryptophan metabolite kynurenine [41], heme [42], and ABTS [15]. It is thus possible that anti-A1M antibodies that recognize degraded radicals bound to urinary A1M, can detect the same epitopes on the 75, 66 and 55 kDa bands seen in Fig. 3. Interestingly, protein-linked kynurenine has been found in the eye [43] supporting this hypothesis.

Anti-oxidants are not yet part of the armamentarium in clinical RRD treatment, but ROS scavengers have been found to be of significant value in patients with acute ischemic stroke [44]. Oxidative stress-related cell death associated with reperfusion after ischemia has been well described in the retina [45]. Similarly, re-attachment of the detached retina is associated with several pathological events, although the underlying molecular mechanisms have not yet been fully understood [46]. Since oxidative stress-related injury

in experimental RD can be significantly attenuated with ROS scavenger treatment [26, 27], this avenue may also be applicable to the clinical situation.

A1M has previously been found to have protective effects against heme- and ROS-induced damage on cells and matrix (reviewed in [6]). Thus, A1M prevented intracellular oxidation, cell-death and up-regulation of cell cycle regulatory and antioxidation genes induced by ROS in the erythroid cell line K562 [3], and silencing of the endogenous A1M expression by addition of siRNA led to an increased cytosol oxidation [3]. Similar results were obtained in primary keratinocytes and using *ex vivo* skin explant cultures [4]. In the skin explants, protection and repair of collagen fibers in extracellular matrix by A1M was also shown, using biochemical methods and electron microscopy [4]. The documented protective antioxidation properties together with the apparent involvement in the response against RRD-related oxidative stress present here makes A1M an intriguing candidate for local or systemic use as adjuvant treatment in conjunction with surgery.

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FIGURE LEGENDS

Figure 1. SDS-PAGE of vitreous samples under reducing conditions. All samples were centrifuged 20 minutes at 14,000 g prior to SDS-PAGE. Lane 1, marked "P", was loaded with 5 µl human plasma diluted 100 x and lanes 2-7 were loaded with 5 µl vitreous body from human patients. Sample loading buffer contained mercaptoethanol. The numbering (No. R3, R4, R5, R6, R12,) refers to the sample numbers in Table 2. MH8 refers to MH sample no. 8.

Figure 2. Comparison of detachment characteristics and vitreous concentrations of A1M and carbonyl groups. The 14 RD samples were subgrouped according to detachment characteristics (vitreous hemorrhage, extent of detachment, macular status) and correlated to A1M and carbonyl groups as described in Materials and Methods. The whole RD sample group (n=14) was also compared to the whole MH control group (n= 14) (right).

Figure 3. Western blotting with anti-A1M. Sample loading buffer contained mercaptoethanol. Lane marked "P" is loaded with 4 µl human plasma diluted 50 x. 3 µl vitreous body sample was added to lane 2-7. A polyclonal antibody (K:107) directed against A1M, diluted 2000 x, was used as primary antibody, and ¹²⁵I-labelled goat anti-rabbit IgG (0.5 x 10⁶ cpm/ml) as secondary antibody. Sample numbers refer to sample IDs in Table 2. MH8 refers to MH sample no. 8. The identification of the A1M-forms as indicated was done according to ref. no [12].

Figure 1

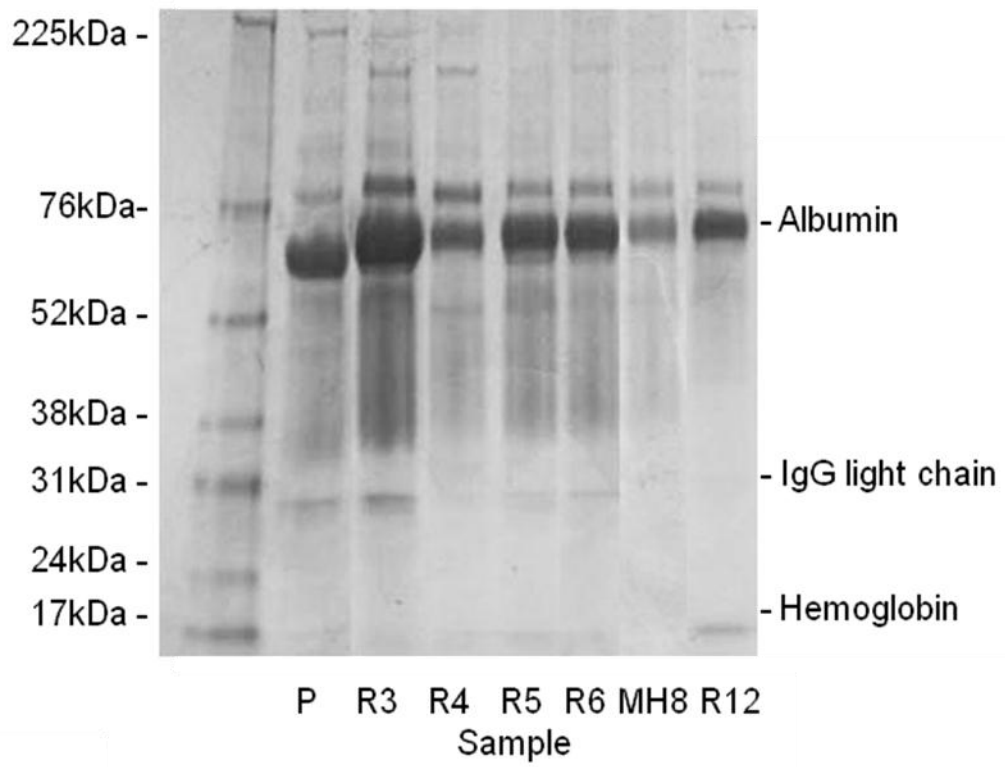


Figure 2

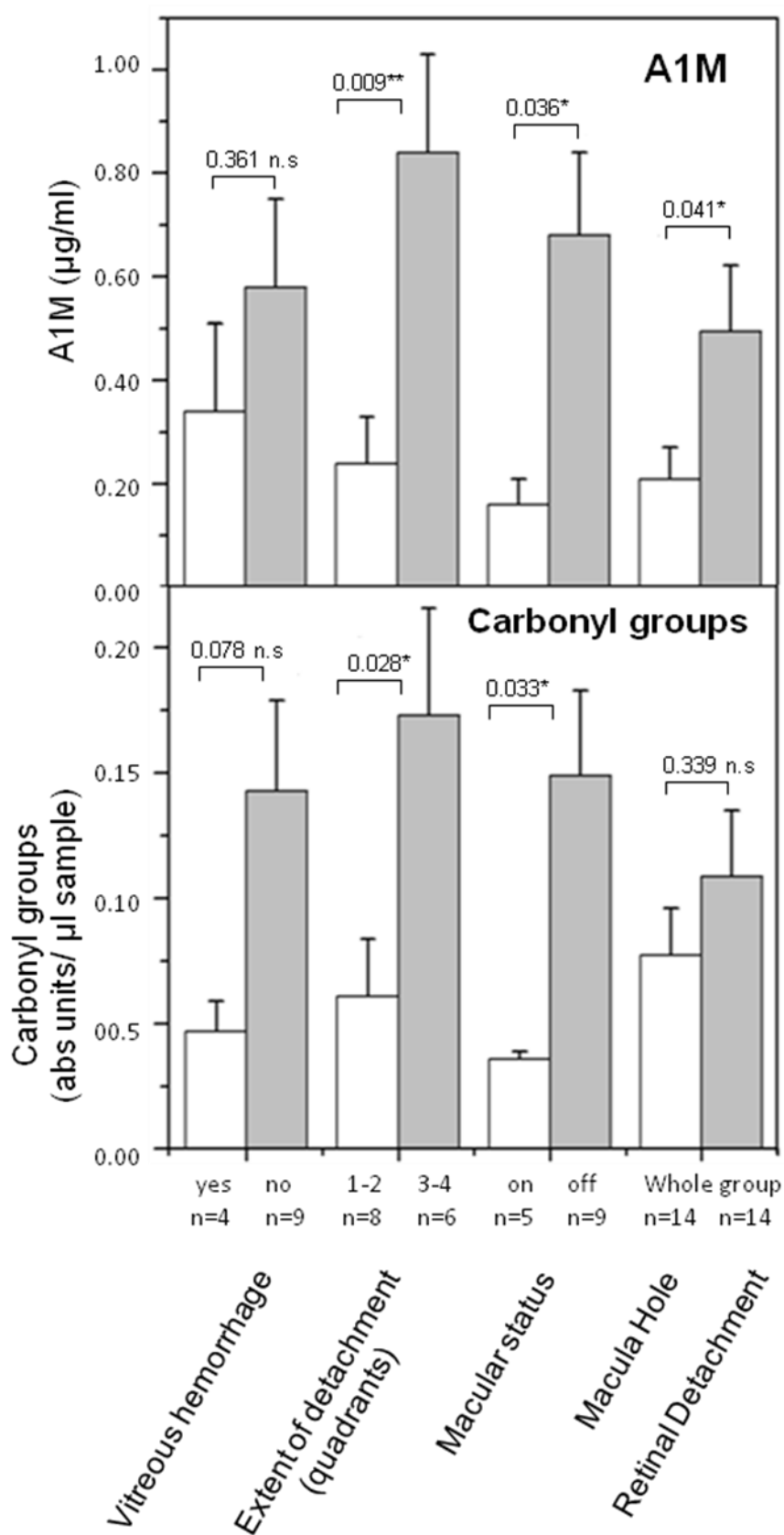
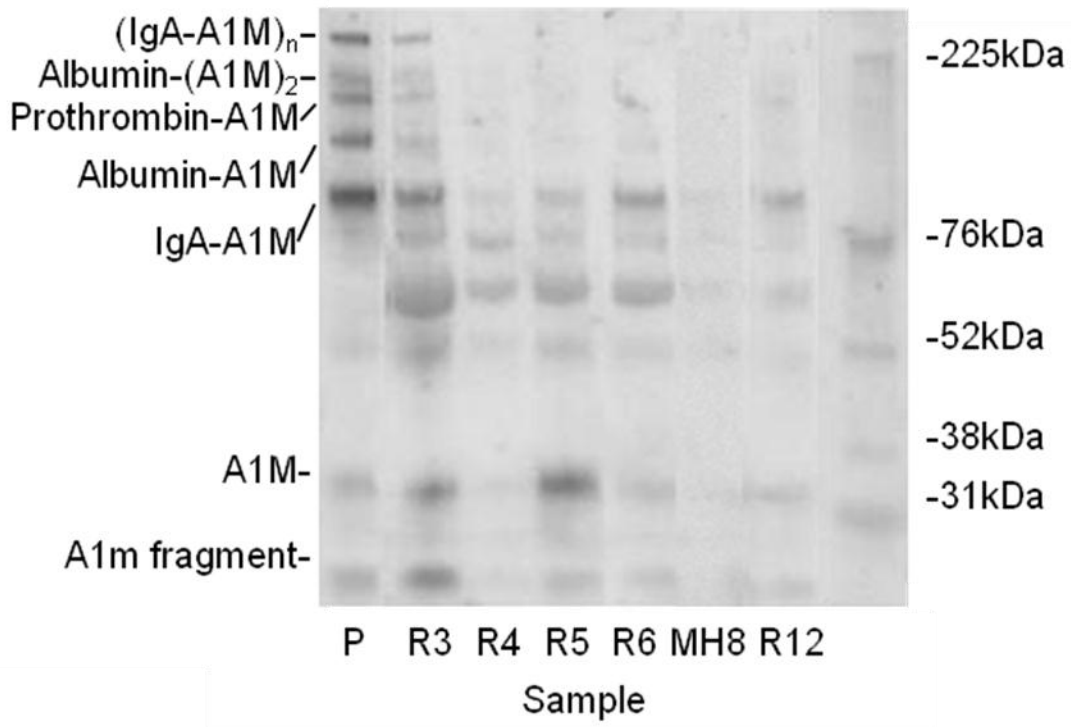


Figure 3



TABLES

Table 1. Preoperative background data for RRD patients (n=14). Items are presented as categorical data with absolute frequencies or as numerical data with mean \pm SEM.

| Variable | Data | Mean \pm SEM | Freq. |
|----------------------------------|--------------|----------------------------------|--------------|
| Age (years) | All: | 59.7 \pm 3.2 | |
| Sex | Male | | 8 |
| | Female | | 6 |
| Preoperative VA (logMAR) | All: | 1.19 \pm 0.3 | |
| Lens status | Phakic | | 11 |
| | Pseudophakic | | 3 |
| Vitreous haemorrhage | Yes | | 5 |
| | No | | 9 |
| Extent of detachment (quadrants) | 1-2 | | 8 |
| | 3-4 | | 6 |
| Macular status | On | | 5 |
| | Off | | 9 |

Table 2. Individual results of the vitreous sample analysis

| Sample no. | ^aDisease | ^bHemoglobin | ^cTotal Protein (mg/ml) | A1M (µg/ml) | Carbonyl groups (abs units/µl sample) |
|-------------------|----------------------------|-------------------------------|--|--------------------|--|
| R1 | RRD | 0 | 1.43 | 0.85 | 0.174 |
| R2 | RRD | + | 2.09 | 1.27 | 0.255 |
| R3 | RRD | + | 1.57 | 0.54 | 0.178 |
| R4 | RRD | 0 | 0.18 | 0.15 | 0.030 |
| R5 | RRD | + | 0.67 | 0.98 | 0.074 |
| R6 | RRD | + | 0.23 | 0.35 | 0.026 |
| R7 | RRD | 0 | 0.26 | 0.11 | 0.029 |
| R8 | RRD | ++ | 0.37 | 0.13 | 0.034 |
| R9 | RRD | 0 | 0.47 | 0.15 | 0.038 |
| R10 | RRD | 0 | 0.18 | 0.05 | 0.021 |
| R11 | RRD | ++ | 1.70 | 1.02 | 0.176 |
| R12 | RRD | ++ | 0.38 | 0.13 | 0.036 |
| R13 | RRD | 0 | 0.17 | 0.03 | 0.023 |
| R14 | RRD | 0 | 1.18 | 1.20 | 0.126 |
| Mean ^d | RRD (n=14) | | 0.78 (0.18) | 0.50 (0.12) | 0.087 (0.021) |
| Mean ^d | MH (n=14) | | 0.52 (0.18) | 0.21 (0.061) | 0.062 (0.014) |

^aRRD = Rhegmatogenous Retinal detachment, MH = Macula hole.

^bSemi-quantification of hemoglobin as seen in SDS-PAGE (Fig. 1): “0” = negative, “+” = low levels, “++” = medium levels, “+++” = high levels

^cVitreous levels of total protein measured by Bradford assay as described in Materials and Methods.

^dMean and (SEM) values are given.

Table 3. A1M and GAPDH mRNA expression in rat retina.

| | GAPDH (Mean Ct \pm SEM) | A1M (Mean Ct \pm SEM) | ^cA1M ($\Delta\Delta$ Ct \pm SEM) |
|--------------------------------|-------------------------------------|-----------------------------------|--|
| ^a Tissue (n=14) | 15.85 \pm 0.26 | 29.80 \pm 0.14 | 0 \pm 0.22 |
| ^b Culture (n=10) | 24.44 \pm 0.16 | 36.12 \pm 0.13 | 2.27 \pm 0.18 |

^aTo explore A1M expression in the non-cultured normal retina tissue, neuroretinas were kept for 3h in PBS as described in Materials and Methods prior to mRNA analysis.

^bTo investigate the capacity of the retina to upregulate A1M under stress-induced conditions, neuroretinas was cultured for 24h as described in Materials and Methods prior to mRNA analysis.

^cCt-values were re-calculated to Δ Ct-values by normalizing to the Ct-values of human glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The $\Delta\Delta$ Ct-values shown in the Table were then calculated by normalizing 24 h-cultured retinas against 3h-incubated retinas. Hence, the $\Delta\Delta$ Ct values of the 3 h-incubated retinas correspond to zero. A lower Ct-value corresponds to an increased mRNA-level and is therefore depicted as an increased $\Delta\Delta$ Ct-value, and vice versa.