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LABORATORY STUDY

Subretinal fluid from rhegmatogenous retinal detachment and blood induces the expression of ICAM-1 in the human retinal pigment epithelium (ARPE-19) *in vitro*

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Abstract

Purpose **To evaluate the effect of subretinal fluid (SRF) from rhegmatogenous retinal detachment (RRD) and blood on the expression of intercellular adhesion molecule-1 (ICAM-1) in the retinal pigment epithelium.**

Methods **The study included 22 patients who had experienced RRD within 1 month before the study and 14 patients with macular holes or pucker. SRF was collected during surgery to repair RRD and the vitreous was collected during vitrectomy. The SRF was cocultured with ARPE-19 cells with and without glucosamine sulphate (GS) and triamcinolone acetonide (TA). Blood from peripheral veins and blood components (red blood cells, platelet-poor plasma, and platelet-rich plasma) were also cocultured with ARPE-19 cells. Vitreous samples were cocultured with ARPE-19 cells in the control. The expression of ICAM-1 was detected and quantified by using flow cytometry.**

Results **The expression of ICAM-1 in RPE cells was significantly higher ($P<0.05$) after 24-h incubation of 40% SRF with ARPE-19 cells. In addition, the expression of ICAM-1 in retinal pigment epithelium (RPE) cells significantly increased ($P<0.01$) when cocultured with blood and blood components. However, there were no differences ($P>0.05$) in ICAM-1 expression when RPE cells were cocultured with or without GS or TA.**

Conclusions **SRF and blood enhanced the expression of ICAM-1 in RPE cells in this study and the increased expression of ICAM-1 by SRF is not inhibited by GS or TA.**

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Introduction

Rhegmatogenous retinal detachment (RRD) is the separation of the photoreceptors from the retinal pigment epithelium (RPE) cell layer that occurs during a retinal break, and allows pathologic subretinal fluid (SRF) to accumulate in the space.[1] The origins of SRF are still a matter of debate, and SRF has been reported as arising from the vitreous,[2,3] serum,[4,5] and retina.[6,7] Most SRF is absorbed after the retina is reattached and the RRD is resolved. However, some patients develop proliferative vitreoretinopathy (PVR) following retinal surgery. PVR is a process of cellular proliferation and contraction, and is the most common cause of failure when the primary RRD has been appropriately treated.[8,9] PVR might develop as an inflammatory and scarring response,[10–12] but whether RRD is also an inflammatory response has not been established.

Intercellular adhesion molecule-1 (ICAM-1) is a transmembrane glycoprotein of 505 amino acids with a molecular weight ranging from 80 to 114 kDa, depending on the degree of glycosylation.[13] It belongs to the immunoglobulin supergene family and contains five immunoglobulin-like domains (D1–D5) that function in cell–cell and cell–matrix adhesion interactions. In general, ICAM-1 appears to play a critical role in immune and inflammatory responses, such as the trafficking of inflammatory cells, in leucocyte effector functions, in the adhesion of antigen-presenting cells to T lymphocytes during antigen presentation, and other responses that have been reported in the literature.[14,15]

In inflammatory responses of the eye, RPE cells may play an important role in the immunogenic process by acting as antigen-presenting cells.[16] Moreover, ICAM-1 was detected in the RPE cells of patients with posterior uveitis, and increased levels of ICAM-1 promoted extravasation of inflammatory cells into the retina.[17]

Glucosamine sulphate (GS) is a naturally occurring amino sugar that exerts immunosuppressive effects *in vitro* and *in vivo*,[18,19] and is widely used in the treatment of osteoarthritis. Recently, GS has been shown to inhibit cytokine-induced ICAM-1 expression in RPE cells *in vitro*.[20] Triamcinolone acetonide (TA) is a synthetic corticosteroid that is widely used in the treatment of ocular diseases. The mechanism of TA action is thought to be through the inhibition of arachadonic acid leading to a decrease in the levels of prostaglandins and leukotrienes—two important mediators of the inflammatory response.[21] In a previous study, it was demonstrated that TA downregulated cytokine-induced expression of ICAM-1 in human choroidal endothelial cells.[22]

We conducted this study to evaluate whether SRF from RRD and blood induce the expression of ICAM-1 in RPE cells. We also evaluated if two anti-inflammatory drugs, GS and TA, inhibit SRF-induced ICAM-1 expression in RPE cells.

Materials and methods

Patients

SRF was collected during the surgical treatment of scleral buckling from 22 eyes from 22 patients diagnosed with RRD. Of the 22 patients, 10 were assigned to undergo acupuncture of peripheral veins, from which fresh blood was collected. The vitreous was used as the control group due to the absence of SRF in normal eyes. The vitreous samples were collected from 14 eyes of 14 patients undergoing three-port vitrectomies for the treatment of macular hole or macular pucker. GS and TA were, respectively, studied in SRF samples randomly collected from 8 and 10 patients. Any patients having systemic diseases (such as diabetics or hypertension), ocular diseases (such as glaucoma or uveitis), or past ocular surgeries (such as glaucoma filtering or cataract surgery) were excluded. Overall, a total of 36 patients, all of whom had visited the Tri-Service General Hospital between April 2006 and March 2007, were included in the study. Informed consent was obtained from all patients after the nature and possible consequences of the study were explained to them. The study abided by the principles of the Declaration of Helsinki and followed the regulations of the hospital's ethics committee.

Sample collection

A sterile syringe was used to collect undiluted SRF by using a 27-gauge needle and manual suction at the sclera puncture site during the procedure of scleral buckling. The collected SRF was transferred to 1.5 ml sterile tubes and was immediately centrifuged at $5000 \times g$ for 15 min at 15°C. The supernatants were aspirated into sterile tubes and stored at −70°C until used. Undiluted vitreous was collected during conventional three-port vitrectomy by using manual suction before opening the infusion line. The vitreous samples were injected into sterile tubes and stored at −70°C until used. Whole blood (WB) was collected by using routine acupuncture of peripheral veins from 10 of 22 patients with RRD. Coagulation was prevented by using anticoagulation citrate dextrose-A solution. The WB samples were transferred to the laboratory immediately. Red blood cells (RBC), platelet-rich plasma (PRP), and platelet-poor plasma (PPP) were obtained by centrifugation of the WB by methods reported in the literature.[23]

Cell culture and treatment

ARPE-19 cells were used in the study and were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). They were maintained in Dulbecco's modified Eagle's medium (DMEM)/F-12 supplemented with 4 mM L-glutamine, 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 mg/ml streptomycin, and 0.25 mg/ml amphotericin B at 37°C in 5% CO_2 in air. In each experiment, RPE cells were grown to confluence, made quiescent for 24 h in the DMEM/F-12 medium without serum, and then stimulated at different times or concentrations of SRF, WB, PRP, PPP, and vitreous as described in the figure legends. When evaluating the effects of GS and TA, cells were preincubated with these drugs for 1 h, and the compounds were present in the growth medium for the duration of the experiments.

Flow cytometry

ICAM-1 expression in ARPE-19 cells was examined by using flow cytometric analysis. Vehicle-stimulated RPE cells were trypsinized and then washed in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and were then resuspended in PBS at 5×10^6 cells/ml. A 100-μl portion of cell solution, at a concentration of 0.5×10^6 cells/ml, was subjected to immunofluorescence staining. This procedure involved sequential 30 min incubations of the cells in 200 ml of wash PBS containing primary anti-human ICAM-1 antibodies (1 : 1000 dilution; Santa Cruz Biotech) and 100 ml of wash PBS containing secondary fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit antibodies (1 : 1000 dilution; Santa Cruz Biotech) at 4°C. Flow cytometry was performed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA) and ARPE-19 cells were quantified based on light scatter. A histogram of fluorescence distribution was constructed, with the fluorescence intensity displayed on the abscissa and the number of cells on the ordinate. The relative mean fluorescence intensity was determined from the histogram as an index of cell-surface expression.

Statistical analysis

To compare data between three or more groups, one-way analysis of variance (ANOVA) was performed followed by using the Bonferroni test. Data are expressed as mean ± SEM and $P < 0.05$ was considered significant.

Results

The demographic characteristics of each treatment group are shown in Table 1. The control group included eight patients with macular holes and six patients with macular pucker. Patients with PVR were excluded from the study.

Effects of SRF and blood on ICAM-1 expression

ARPE-19 cells were cocultured with vitreous samples for 24 h as the control group. Based on a preliminary study that found no significant differences in ICAM-1 expression due to varied concentrations of vitreous, SRF, and blood samples (data not shown), all samples were diluted to 40% in this study. Background relative mean fluorescence intensity for ICAM-1 expression was measurable but small in vehicle-treated control cells. The expression of ICAM-1 was significantly higher in cells incubated with SRF ($P < 0.05$) and WB ($P < 0.01$) than it was in the control group. ICAM-1 expression in ARPE-19 cells incubated with the three components of WB (RBC, PPP, and PRP) was significantly higher than that in the control group (Figure 1). Furthermore, incubation of the cells with RBC and PRP resulted in significantly higher ($P < 0.05$) expression of ICAM-1 than when the cells were incubated with SRF or PPP.

Effects of GS on ICAM-1 expression

To evaluate whether GS inhibits the expression of ICAM-1 induced by exposure to 40% SRF, cells were incubated for 1 h with three concentrations of GS (10 mg/l, 100 mg/l, and 1000 mg/l) before the addition of SRF for

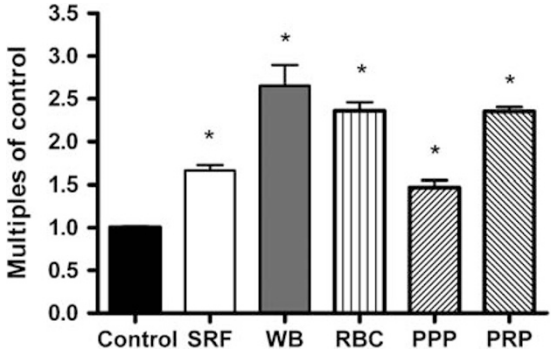

Figure 1 SRF, WB, RBC, PPP, and PRP all increased ICAM-1 expression in ARPE-19 cells. *Statistical significance compared with control ($P < 0.05$).

Table 1 Demographic characteristics

	Group				
	Control	*SRF*	*Blood*	*GS*	*TA*
Number	14	22	10	8	10
Age (year)	50.2 ± 8.1	47.3 ± 13.8	43.7 ± 17.2	54.6 ± 7.0	51.2 ± 12.9
Sex (male/female)	9/5	16/6	8/2	6/2	7/3
Eye (right/left)	8/6	12/10	—	4/4	5/5
RD duration (day)	—	12.1 ± 7.5	—	—	—

Abbreviations: SRF = subretinal fluid; GS = glucosamine sulphate; TA = triamcinolone acetonide; RD = retinal detachment.

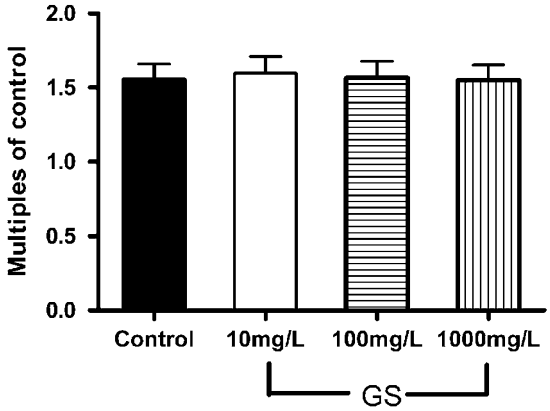

Figure 2 Effects of GS on ICAM-1 expression in SRF-stimulated ARPE-19 cells.

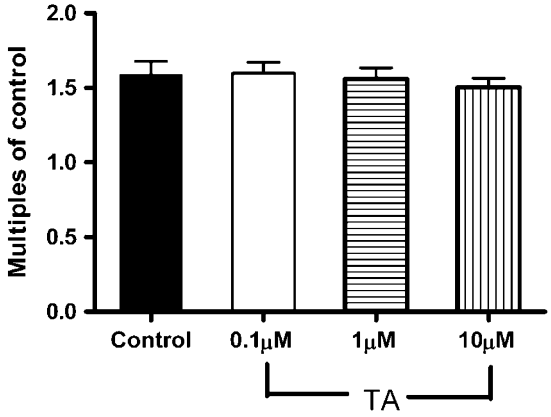

Figure 3 Effects of TA on ICAM-1 expression in SRF-stimulated ARPE-19 cells.

24 h. None of the tested concentration of GS significantly inhibited ICAM-1 expression (Figure 2).

Effects of TA on ICAM-1 expression

Three concentrations of TA (0.1 μM, 1.0 μM and 10 μM) were used to evaluate whether TA inhibits ICAM-1 expression. Cells were incubated for 1 h before the addition of 40% SRF for 24 h. As was observed with GS, these concentrations of TA did not significantly inhibit ICAM-1 expression (Figure 3).

Effects of RD duration on ICAM-1 expression

To assess whether SRF exposure resulting from differing durations of RRD induce a varied response in ICAM-1 expression, study participants were divided into four groups based on the duration of the RRD: 1–3 days, 4–7 days, 7–14 days, and 15–28 days. Although the ICAM-1 expression seemed higher with an increase in the duration of RRD, no statistical correlation was found (Figure 4).

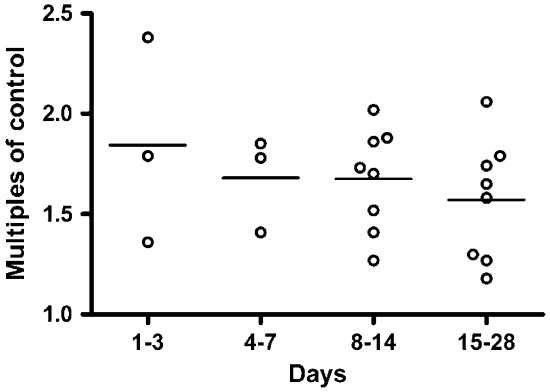

Figure 4 ICAM-1 expression by SRF following varied durations of RRD.

Conclusion

SRF is a liquefied vitreous that flows through the retinal break and into the subretinal space in the early stages of RRD. Breakdown of the blood–retinal barrier following RRD results in the leakage of blood, which may promote an inflammatory reaction and the proliferation of retinal glial and RPE cells.[24,25] In previous studies, various factors involved in the inflammation process, such as gelatinase activity,[26,27] connective tissue growth factor,[28] pigment-epithelium-derived factor,[29] IL-6,[30] s-100,[31] and TGF-β2[25] have been reported to be present in SRF. In our study, ARPE-19 cells cocultured with SRF showed elevated expression of ICAM-1 *in vitro*. This demonstrates that some component of SRF is capable of inducing the expression of ICAM-1 in RPE cells. ICAM-1 acts as an inflammatory factor and plays a critical role as an adhesion or a signal transduction molecule in the inflammatory response.[15] It had also been linked to ocular inflammatory diseases, such as uveitis.[32] In other words, SRF may induce ocular inflammation if RRD persists. If left untreated, RRD may increase the chances of forming PVR.

RPE cells have been implicated in the pathogenesis of ocular inflammatory diseases, such as PVR or certain types of posterior uveitis. In these disorders, RPE cells proliferate in the vitreous cavity and on the retinal surface and undersurface, where they may form contractile membranes and produce inflammatory mediators that recruit and activate inflammatory cells.[33,34] Increased levels of proinflammatory cytokines, such as TNF-α, IFN-γ, or IL-1β, that could induce ICAM-1 expression have been reported in patients with PVR.[35–37] A previous study that compared the vitreous levels of ICAM-1 between patients with a high risk of PVR and

those with a lower risk indicated that ICAM-1 expression was a risk indicator for PVR in RRD.[38] However, our data provide additional information indicating that ICAM-1 expression in RPE cells is elevated following RRD, even when RRD has not progressed to PVR.

ICAM-1 expression in RPE cells cocultured with fresh blood (WB) was higher than that in cells incubated with SRF alone. This indicates that blood can induce a more severe inflammatory response in RPE cells than can SRF. Previously, Vergara *et al*.[39] reported that intravitreal injection of blood or ferrous chloride solutions in rabbits' eyes induced severe inflammation and that iron was an important stimulus for intraocular inflammation. Baudouin *et al*.[40] reported that intravitreal injection of PRP in rabbits' eyes caused the animals to develop an experimental mode of PVR and that platelets also play a role in intraocular inflammation. Therefore, we evaluated the role of three major components of blood in intraocular inflammation. Not surprisingly, all three components—RBC, PPP, and PRP—increased ICAM-1 expression in RPE cells, but no synergistic effect was apparent. Furthermore, RBC and PRP caused higher induction of ICAM-1 expression than PPP. This is in agreement with the experimental induction of the animal mode of uveitis or PVR, which can be induced by intravitreal injection of PRP or blood.

Our data showed that blood or its components may induce ocular inflammation *in vitro*. However, simple vitreous haemorrhage seems not to result in severe ocular inflammation clinically, and most doctors recommend conservative treatment in the condition. We thought this was because a simple vitreous haemorrhage may not disturb the integrity of the retina and that RPE cells might not come into contact with blood. On the other hand, RRD in patients may be complicated by the presence of vitreous haemorrhage, and the operative choice between early vitrectomy and cryopexy with scleral buckling is difficult. Our data may provide information that allows a more informed operative decision. Blood and its components induced more ocular inflammation, and early vitrectomy may be indicated to reduce the chances of PVR. Further investigation of this possibility is needed.

GS is a popular nutritional supplement in relieving the symptoms of osteoarthritis[41–44] but is not used clinically for the treatment of ocular diseases. TA is a synthetic corticosteroid and has been used to control intraocular inflammatory conditions since 1974.[45] Both drugs have anti-inflammatory effects and they had been reported to inhibit cytokine-induced ICAM-1 expression *in vitro*.[20–22,46,47] Therefore, our study also evaluated the effects of the two drugs on ICAM-1 expression induced by SRF. The results were negative; neither drug significantly reduced ICAM-1 expression in RPE cells stimulated by SRF. In past studies, TNF-α or IFN-γ were used to stimulate the expression of ICAM-1 and GS or TA countered the actions.[20–22] However, they may not be major components of SRF, and this could be the reason for the different results. In our SRF array (data not shown) and a past report,[30] IL-6 was the major component of SRF, and the ICAM-1 expression pathway induced by IL-6 in RPE cells may be different from those induced by TNF-α or IFN-γ. GS had been shown to downregulate ICAM-1 expression through the prevention of NF-κB activity and GS-activated STAT1 nuclear translocation in RPE cells,[20] but SRF may not induce ICAM-1 expression by the same pathway.

After a tissue injury, a cascade of mechanisms occurs to repair the wound. Wound healing may progress following RRD, and the components of SRF may vary with time. van Setten *et al*.[28] has reported that the concentrations of connective tissue growth factor in SRF changed over time, and La Heij *et al*.[48] has stated that subretinal cGMP levels were moderately correlated with the duration of the retinal detachment. Bakunowicz-Lazarczyk *et al*.[30] noted that the concentration of IL-6 in SRF was significantly higher 5–8 weeks following RRD. However, in our study, the expression of ICAM-1 did not differ based on the duration of exposure to SRF. A possible reason was that few samples were available for exposure durations of less than 1 week; there were only three samples for each exposure duration (0–3 and 4–7 days), compared with eight samples for the other groups.

In conclusion, SRF exposure resulting from RRD induced the expression of ICAM-1 in RPE cells *in vitro*, and WB, RBC, and PRP enhanced the phenomenon. The elevated expression of ICAM-1 by SRF cannot be suppressed by GS or TA. Furthermore, the effect of SRF induction of ICAM-1 expression did not differ based on RRD duration. We demonstrated that ICAM-1 had been expressed even when RRD has not yet progressed to PVR. Early removal of blood may be necessary in RRDs combined with vitreous haemorrhage because of higher inflammation induced by blood and its components.

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