TLR4 inhibitor attenuates amyloid-β-induced angiogenic and inflammatory factors in ARPE-19 cells: Implications for age-related macular degeneration

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Abstract. Subretinally-deposited amyloid-β (Aβ) is an important factor in age-related macular degradation (AMD) often leading to irreversible blindness in the elderly population. The molecular mechanism underlying Aβ deposition during AMD remains unclear. The expression of inflammatory and angiogenic factors was examined by treatment of retinal pigment epithelial (RPE) cells with the oligomeric form of Aβ (OAβ1-42). Changes in the mRNA expression levels of various cytokines was detected by the QuantiGenePlex 6.0 Reagent system, and the protein expression level was determined by western blotting. Culture supernatants were detected using a multiplex cytokine assay and enzyme-linked immunosorbent assays. The *in vitro* tube formation was evaluated by a Matrigel assay. The present study highlights that OAβ1-42 activates the toll-like receptor 4 (TLR4), myeloid differentiation factor 88 and phosphorylation nuclear factor-κB signaling pathway in RPE cells. Additionally, it increased the mRNA and protein expression of interleukin (IL)-6, IL-8, IL-33, vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and angiopoietin 2. Furthermore, the TLR4 inhibitor (COBRA) attenuated the expression of inflammatory and angiogenesis factors, particularly IL-6, IL-8, IL-33, bFGF and VEGF. When human umbilical vein endothelial cells (HUVECs) were co-cultured with the COBRA-treated RPE cell culture supernatant the length of the endothelial cell network (measured by calculating tip cell lengths of endothelial cells) was impaired when compared with the HUVECs that were co-cultured with the cell supernatant exposed to OAβ1-42. These results suggest that the TLR4-associated pathway may be a potential target for the treatment of AMD.

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Introduction

Age-related macular degeneration (AMD) is the leading cause of blindness in the elderly population, as it results in a loss of vision due to damage to the retina. Based on clinical findings, AMD may be divided into 'dry' and 'wet' AMD (1,2). Drusen are often used to diagnose AMD, they are also associated with the geographic atrophy of the retinal pigment epithelial (RPE) cells in dry-AMD and increase the risk of developing the exudative form of AMD (wet-AMD) (3,4). Amyloid-β (Aβ) is an important constituent of drusen and is primarily associated with neurodegenerative processes in the brain during Alzheimer's disease (AD) (5,6). Previous studies indicated that Aβ is also an important contributor to the progression of AMD (3,7).

The Aβ is a peptide that is ubiquitously and normally expressed in humans in two forms of 39- and 43-amino acids in length (termed Aβ40 and Aβ42, respectively) (8,9). Aβ42 is associated with AD plaques that are composed of a multitude of highly aggregated Aβ fibrils, and represent abnormal pathological lesions (10). Aβ40, the more common and less harmful form, is present in drusen and activates the inflammatory response in RPE cells aiding in AMD progression (11). Bruban *et al* (12) reported that the oligomeric form of Aβ1-42 (OAβ1-42), is toxic for RPE cells *in vitro* and *in vivo*. Furthermore, subsequent studies demonstrated that Aβ upregulated the inflammasome-associated factors interleukin-1β (IL-1β), IL-18 and caspase-1 (11,13), and cytokines IL-6, IL-8 and tumor necrosis factor-α (TNF-α) (14,15). Additionally, angiogenic factors have also been associated with Aβ in AD. Ets-1, an angiogenic transcription factor has been identified to co-localize with vascular endothelial growth factor (VEGF) and soluble Aβ in the microvasculature (16). In addition, astrocytes have been shown to be involved with Aβ-induced angiogenesis in rat hippocampal

cells (17). Therefore, Aβ may be responsible for triggering the angiogenic and inflammatory responses involved in the pathogenesis of AMD (14-18).

Toll-like receptor 4 (TLR4) is involved in microbial and autoimmune pathogenesis, as well as chronic inflammatory conditions (19). The TLR4 gene is located on the 9q32-33 chromosome region that harbors a potential AMD susceptibility locus (20,21). TLR4 was also identified to be involved in the phagocytosis of the outer photoreceptor segments in RPE cells (22). Impairing phagocytosis in the photoreceptor layer, causes accumulation of lipofuscin fluorophore, which may lead to AMD. Aβ may induce upregulation of TLR4 and nuclear factor-κB (NF-κB) expression in microglia as reported by Zhou *et al* (23). However, the link between the TLR4 signaling pathway and Aβ in RPE cells remains poorly understood.

The current study aimed to determine whether Aβ triggered upregulation of TLR4 and NF-κB expression and whether Aβ activates the release of cytokines and growth factors via the TLR4 and NF-κB signaling pathways in RPE, thus aiding the progression of AMD. The results of the present study provide strong evidence in support of the involvement of Aβ-induced local inflammatory response in RPE cells in the pathogenesis of AMD.

Materials and methods

RPE cell culture and cell treatment. The human ARPE19 cell line [CRL-2302; American Type Culture Collection (ATCC), Manassas, VA, USA] is a non-transformed human RPE cell line that has numerous differentiated properties typical of RPE cells *in vivo*. ARPE-19 cells were cultured in Dulbecco's modified Eagle's medium: F12 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% heat-inactivated fetal calf serum (Gibco; Thermo Fisher Scientific, Inc.). At 80% confluence, the ARPE-19 cultures were treated with 1 mM concentrations of the Aβ peptides (Bachem Distribution Services GmbH, Weil am Rhein, Germany) for 30 min, 1, 3, 6, 12 or 24 h and their respective inactive reverse peptides (Bachem Distribution Services GmbH), which served as controls. All of the cells were used between passage 3 and 7. For the TLR4 inhibitor (COBRA; Novus Biologicals, LLC, San Diego, CA, USA) treatment group, cells were pre-treated with the TLR4 inhibitor, 20 μM COBRA, 4 h prior to being exposed to the Aβ peptides. COBRA interferes with interaction between TIRAP/Mal and the TIR domain of TLR2 or TLR4.

Procedure of Aβ oligomerization. Aβ1-42 (H-1368), Aβ42-1 (H-3976, inactive reverse control peptide of Aβ1-42), Aβ1-40 (H-1194) and Aβ40-1 (H-2972, inactive reverse control peptide of Aβ1-40), were used in the experiments and supplied by Bachem Holding AG (Bubendorf, Switzerland). The preparation of the oligomerization Aβ1-42 has been previously described by Bruban *et al* (12) (technical notes on the solubilization and oligomerization of the Aβ peptides supplied by Bachem Holding AG). Non-oligomerized Aβ1-40 and Aβ1-42 (Bachem Distribution Services GmbH) were incubated at 37˚C for 5 days and stored at -20˚C until they were used. Each aliquot was used only once.

Gene expression of cytokines in ARPE-19 cells using the QuantiGenePlex 6.0 Reagent system. Target-specific RNA molecules of ARPE-19 cells [TLR4: NM_003266; myeloid differentiation factor 88 (MyD88): NM_002468; NF-κB: NM_003998; IL-6:NM_000600; IL-8:NM_000584; IL-33: NM_033439; VEGF: NM_003376; basic fibroblast growth factor (bFGF): NM_002006; angiopoietin 2 (Ang2): NM_001147] were detected using the QuantiGenePlex 6.0 Reagent System according to the manufacturer's protocol (Affymetrix, Inc., Fremont, CA, USA). RNA extracted from cell lysates was captured by fluorescent microspheres (Affymetrix, Inc.). Signals of cascade amplification were detected with the Luminex 100 xMAP system and Bio-Plex software (version 5.0; Bio-Rad Laboratories, Hercules, CA, USA). The geometric means of the two housekeeping genes, peptidylprolyl isomerase B (NM_011149) and hypoxanthine phosphoribosyltransferase 1 (NM_013556) for the ARPE19 cells were used for normalizations. Fold-changes were the relative ratios between the normalized values of the four infected groups and the values of the untreated group. ARPE19 cells from three different cell samples were combined for one detection, and the experiments were repeated three times.

Multiplex cytokine assay. Procarta cytokine profiling kit (Affymetrix, Inc.) was used to simultaneously detect IL-6, IL-8, VEGF, and bFGF in the cell culture supernatants, according to manufacturer's protocol. Antibody beads (50 μl) were added to each well of the filter plate and washed with wash buffer. Then, 50 μl cell culture supernatant was added to each well, incubated for 1 h at room temperature, and washed with wash buffer. Subsequently, 25 μl per well of the detection antibody was added and the filter plate was shaken at 30 x g for 30 min at room temperature. Following the addition of Streptavidin-phycoerythrin, the signals were detected using a Luminex 200 instrument (Bio-Rad Laboratories).

Enzyme-linked immunosorbent assay (ELISA). The levels of IL-33 and Ang2 were determined using Human IL-33 Quantikine ELISA kit (R&D Systems, Inc., Minneapolis, MN, USA) and Human Ang2 Quantikine ELISA kit (R&D Systems, Inc.,) according to the manufacturer's instructions. The 100-μl cell culture supernatant was added to the wells ($1x10^5$ cells/well), which was pre-coated with IL-33 and Ang2 antibodies. The absorbance was measured at 450 nm by microplate reader, model 450 (Bio-Rad Laboratories). All experiments were performed at least three times.

Western blot analysis. RPE cells were harvested and lysed in radioimmunoprecipitation assay buffer (1% Nonidet P-40, 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulfate in phosphate-buffered saline; Affymetrix, Inc.) and centrifuged at 25,000 x g for 15 min at 4˚C. NuPAGE Bis-Tris gels (10%; Invitrogen) were used according to the manufacturer's protocol. The membranes were blocked with 10% fat-free milk and incubated with primary antibodies overnight at 4˚C. The anti-TLR4 antibody, purchased from Abcam (ab22048; Cambridge, MA, USA) was used at a 1:1,000 dilution. The following primary antibodies were purchased from Cell Signaling Technology, Inc. (Beverly,

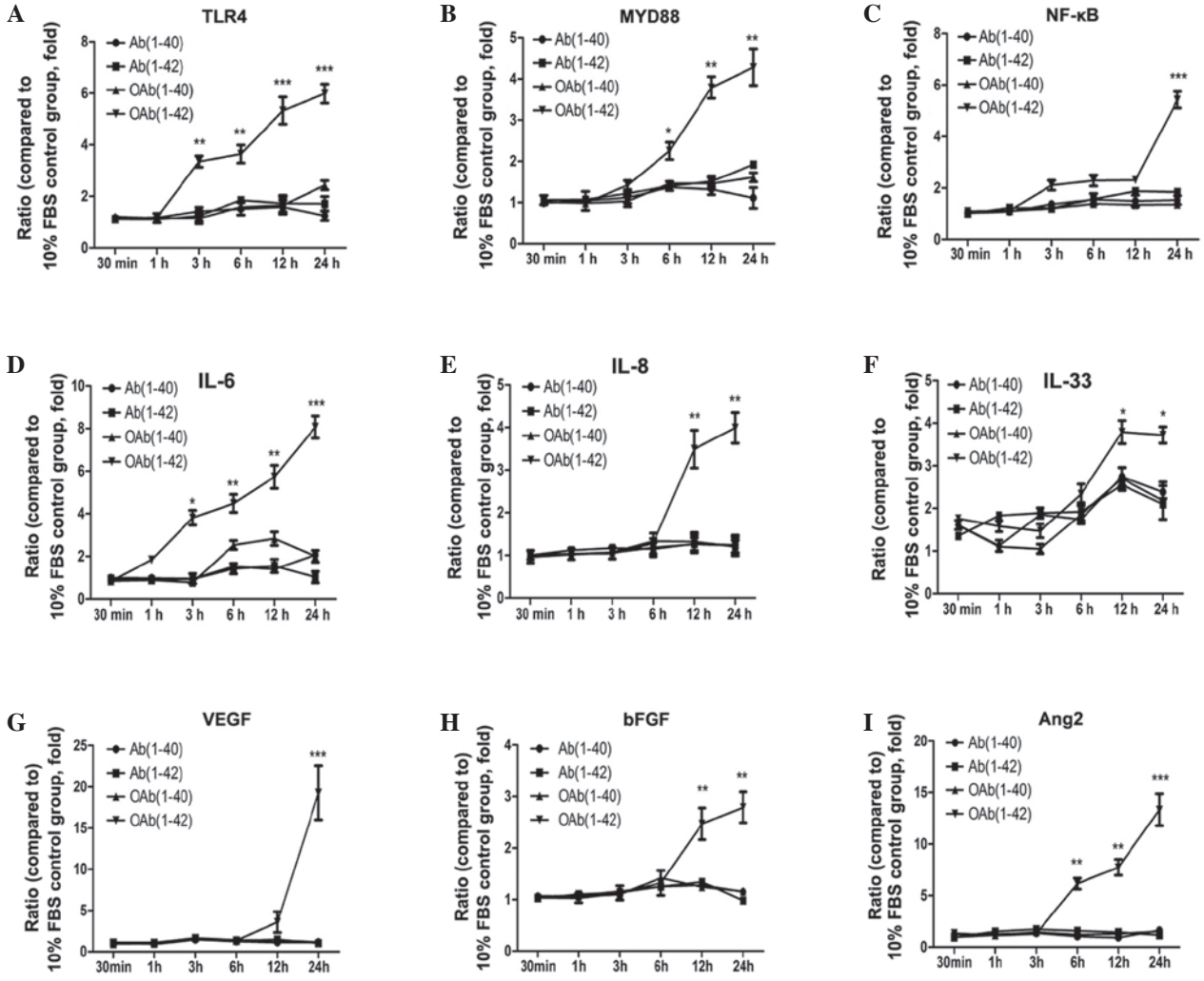

Figure 1. mRNA expression levels of cytokines in RPE cells. QuantiGene assay was used to determine the mRNA levels in RPE cells treated with or without 1 μM OAβ1-42. Fold-changes were the relative ratios of (A) TLR4, (B) MyD88, (C) NF-κB, (D) IL-6, (E) IL-8, (F) IL-33, (G) VEGF, (H) bFGF and (I) Ang2 between the normalized values of the four infected groups and the values of the untreated group. Values are expressed as the mean ± standard error, n=3, *P<0.05, **P<0.01 and ***P<0.001. FSB, fetal bovine serum; OAβ1-42, oligomeric form of Aβ; TLR4, toll-like receptor 4; MyD88, myeloid differentiation factor 88; NF-κB, nuclear factor-κB; IL-6,-8,-33, interleukin-6,-8,-33; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; Ang2, angiopoierin-2; RPE, retinal pigment epithelial.

MA, USA) and were used at a 1:1,000 dilution: MyD88 (cat. no. 4283), phosphorylation-NF-κB (cat. no. 3033), and β-actin (cat. no. 4970). The anti-rabbit (cat. no. 7074) and anti-mouse (cat. no. 7076) horseradish peroxidase-conjugated secondary antibodies (dilution, 1:2,000 for the two; Cell Signaling Technology, Inc.) were applied for 1 h at room temperature. Labeled proteins were detected using an enhanced chemiluminescence western blotting system (Pierce Biotechnology, Inc., Rockford, IL, USA). Image J analysis software (version 1.49; National Institutes of Health, Bethesda, MD, USA) was used to quantify the optical density of each band. Relative changes in protein expression were calculated in relation to normal group and expressed as a fold-change. Each experiment was repeated at least three times.

Tube-formation assay. Human umbilical vein endothelial cells (HUVECs; CRL-1730, ATCC) were used in the current study for *in vitro* evaluation of angiogenesis as previously described (24). All RPE cells were exposed to Aβ; however, two treatment groups were also established, one where the cells were exposed to 20 μM COBRA for 4 h and another where they were not. Subsequently, 200 μl Matrigel (BD Biosciences, San Diego, CA, USA) was added to a 24-well pre-cooled plate and then allowed to polymerize at 37˚C for 30 min. Trypsin-harvested HUVEC cells ($1x10^5$ cells/well) suspended in 300 μl of the fresh assay medium were seeded onto Matrigel and incubated for 8 h. The networks from five randomly selected fields were photographed (Eclipse 50i; Nikon Corporation, Tokyo, Japan). The length of the capillary-like structures, in two-dimensional microscope images, was measured using Image J software (version 1.49). The experiments were repeated three times.

Statistical analysis. Data analysis was performed using the following statistical software programs: Prism version 5.0 (GraphPad Software, Inc., San Diego, CA, USA) and SPSS version 16.0 (SPSS, Inc., Chicago, IL, USA). All data are presented as the mean ± standard error. Data sets were examined by one-way analysis of variance followed by a post hoc

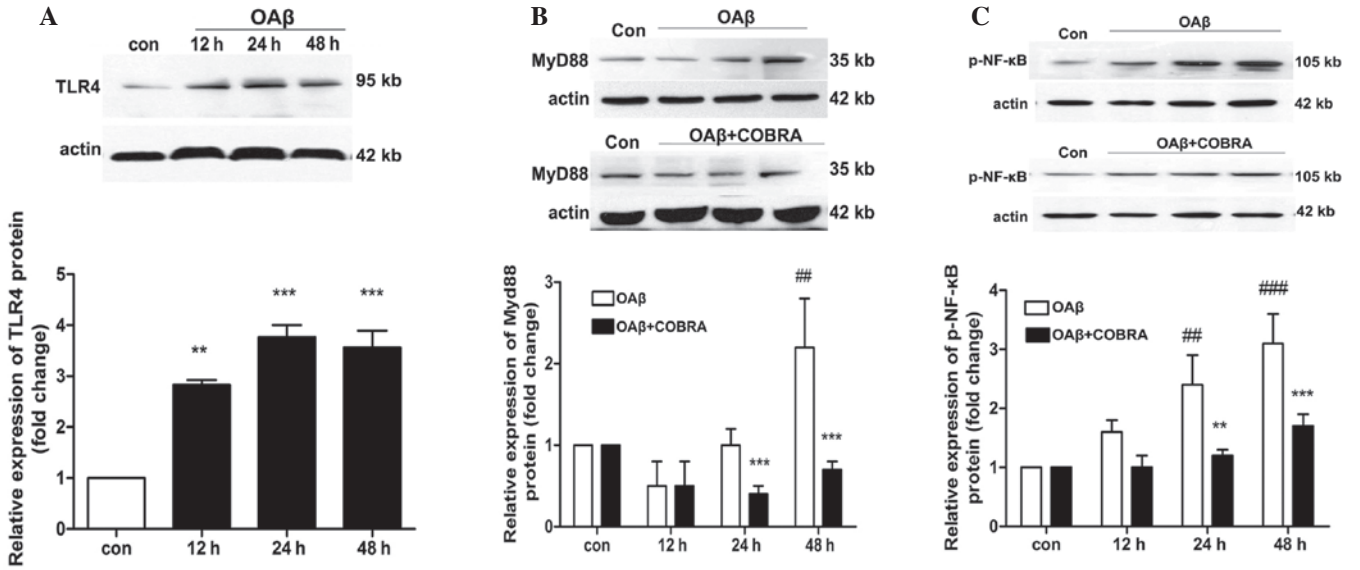

Figure 2. (A) Protein expression and western blotting of TLR4 in RPE cells. Effects of COBRA on the protein expression of (B) MyD88 and (C) p-NF-κB in RPE cells induced by OAβ1-42. Three independent experiments were performed and data are expressed as the mean ± standard error. **P<0.01 and ***P<0.001 vs. control group; $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. OAβ + COBRA group. OAβ1-42, oligomeric form of Aβ; TLR4, toll-like receptor 4; MyD88, myeloid differentiation factor 88; p-NF-κB, phosphorylation-nuclear factor-κB; RPE, retinal pigment epithelial; COBRA, TLR4 inhibitor.

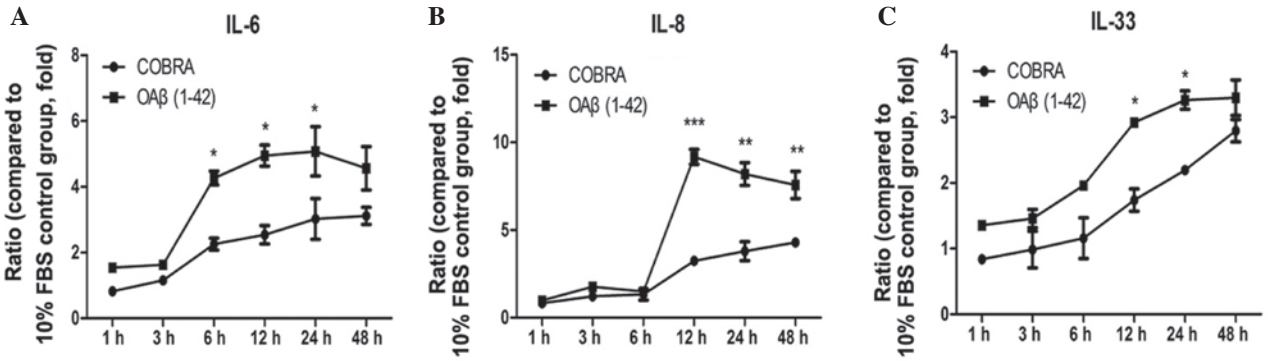

Figure 3. Effects of COBRA on the expression of the inflammatory cytokines (A) IL-6, (B) IL-8 and (C) IL-33 in retinal pigment epithelial cells exposed to OAβ1-42. Three independent experiments were performed and data are expressed as the mean ± standard error. *P<0.05, **P<0.01 and ***P<0.01 vs. COBRA group. FBS, fetal bovine serum; OAβ1-42, oligomeric form of Aβ; IL-6,-8,-33, interleukin-6,-8,-33; COBRA, TLR4 inhibitor.

Dunnett's test. For group comparisons, generalized linear mixed models were used as the present data was measured and obtained at various time-points. P<0.05 was considered to indicate a statistically significant difference.

Results

Different Aβ agents upregulate mRNA levels of cytokines in ARPE19 cells. In the current study, different Aβ agents were used to stimulate ARPE19 cells. The mRNA expression levels of all cytokines was shown in Fig. 1. In this assay, TLR4, MyD88 and NF-κB were upregulated at 3, 6 and 12 h, respectively. Following a 24 h incubation, TLR4, MyD88 and NF-κB increased to 6.0±0.37, 4.3±0.44 and 5.4±0.32-fold, respectively, in the OAβ1-42 group, which was significantly higher than in the other groups (Fig. 1A-C)

As presented in Fig. 1D-F, inflammatory factors were upregulated from 3 and 12 h. IL-6, IL-8, IL-33 increased to 8.1±0.52, 4.0±0.36 and 3.7±0.19-fold, respectively, in the OAβ1-42 group respectively, which was significantly higher than other groups. The mRNA expression of angiogenic cytokines was upregulated from 6 h. VEGF expression was increased to 19.3±3.28 in the OAβ1-42 group, which was significantly higher compared with the other groups after 24 h (Fig. 1G). The mRNA expression level of bFGF increased by 3.0±0.30-fold at 24 h in the OAβ1-42 group (P<0.01; Fig. 1H). Ang2 steadily increased from 6 h onwards (13.3±3.0-fold; Fig. 1I). The expression of TLR2 and TLR3 mRNA was also determined; however, there was no significant difference compared with the control group (data not shown).

OAβ1-42 is involved in the activation of the TLR4 signaling pathway in ARPE19 cells. Western blot analysis detected the protein expression of TLR4, MyD88 and phosphorylation-NF-κB in ARPE19 cells treated with OAβ1-42 (Fig. 2). Fig. 2 indicates that the protein expression of TLR4 increased at 12 h and reached peak at 24 h. The expression of TLR4 was upregulated 3.7-fold compared with

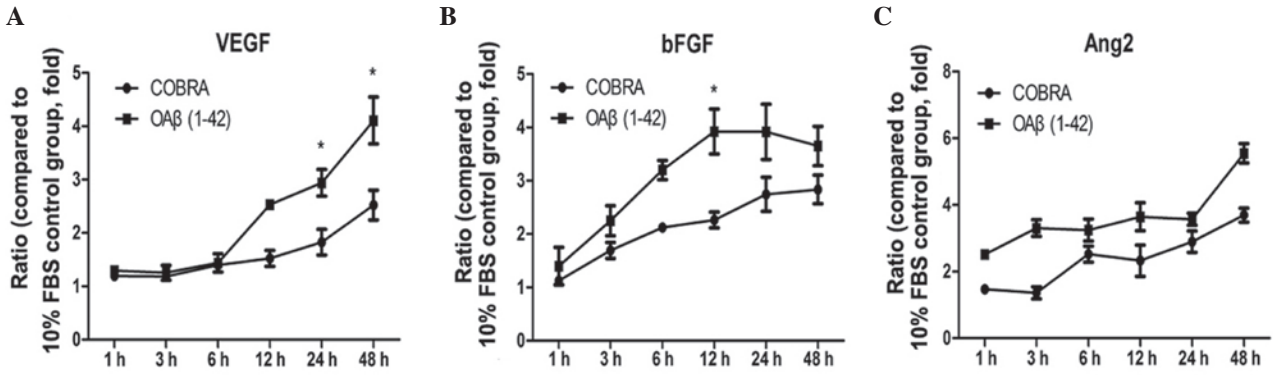

Figure 4. Effects of COBRA on the protein expression levels of angiogenic cytokines (A) VEGF, (B) bFGF and (C) Ang2 in OAβ1-42-treated retinal pigment epithelial cells. Three independent experiments were performed and data are expressed as the mean ± standard error. *P<0.05, vs. COBRA group. FSB, fetal bovine serum; OAβ1-42, oligomeric form of Aβ; COBRA, TLR4 inhibitor; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; Ang2, angiopoierin-2.

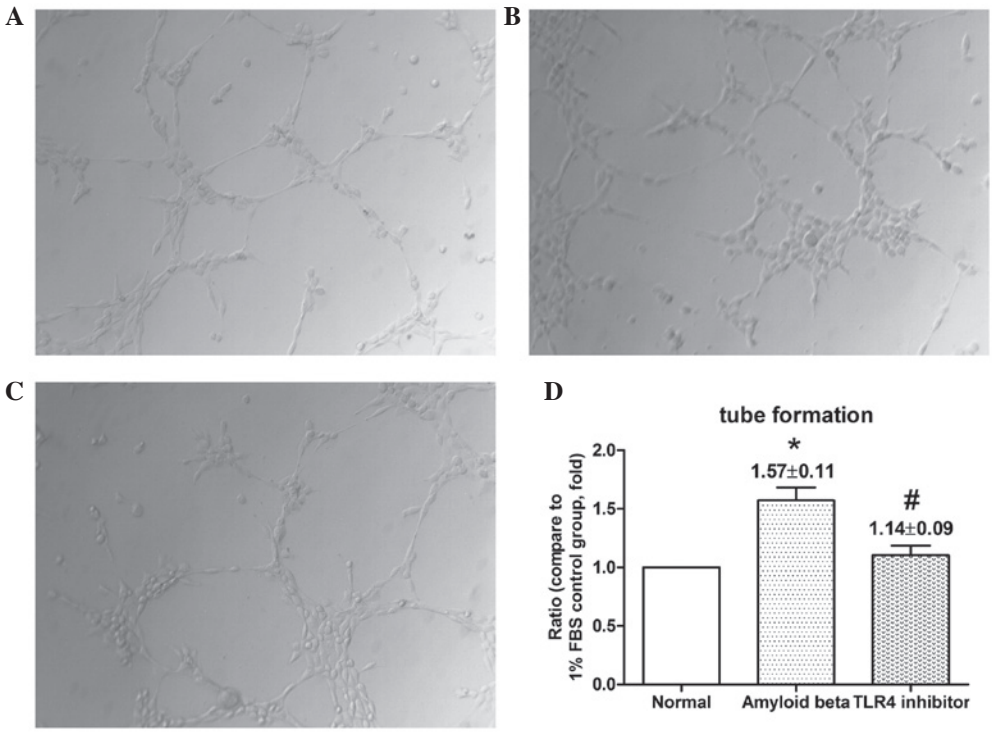

Figure 5. Effect of OAβ1-42 and COBRA on tube formation of human umbilical vein endothelial cells. The images of the angiogenic network in the (A) control group, (B) OAβ1-42 group and (C) COBRA group. Magnification, x100. (D) Quantification of the length of the angiogenic network. Data were determined as network length and shown as the mean percentage control ± standard error. *P<0.05 vs. control; #P<0.05 vs. OAβ1-42 group. FBS, fetal bovine serum; TLR4, toll-like receptor 4; OAβ1-42, oligomeric form of Aβ; COBRA, TLR4 inhibitor.

the control group (Fig. 2A). Similarly, the expression levels of MyD88 and p-NF-κB were also increased in RPE cells treated with OAβ1-42 (P<0.01 and P<0.001 vs. OAβ + COBRA; Fig. 2B and C).

Western blot analysis was also used to determine the effect of COBRA on the protein expression levels of MyD88 and p-NF-κB. As presented in Fig. 2B and C the protein levels of MyD88 and NF-κB were significantly reduced in ARPE19 cells following COBRA treatment when compared with the OAβ1-42 group at 24 and 48 h.

COBRA downregulates the expression of inflammatory and angiogenic factors in ARPE19 cells induced by OAβ1-42. The protein expression level of inflammatory factors increased from 6 h of treatment in the two groups. IL-8 was upregulated by 9.17±0.43-fold at 12 h in the OAβ1-42 group (P<0.001 vs. control). IL-6 and IL-33 increased by 5.1±0.75-fold and 3.3±0.14-fold at 24 h, respectively, in the OAβ1-42 group (P<0.05 vs. control; Fig. 3A and C).

Furthermore, changes in the protein concentration of inflammatory and angiogenic factors in cells treated with COBRA and OAβ1-42 were detected (Figs. 3 and 4). IL-6 and IL-8 expression was significantly reduced at 12 h following COBRA treatment (P<0.05 and P<0.001 vs. the COBRA group, respectively). However, IL-33 expression in the COBRA group was at a similar level as the OAβ1-42 group at

48 h (Fig. 3C). VEGF and Ang2 expression was increased by 4.1±0.44-fold and 5.5±0.29-fold at 4 h, while bFGF, increased by 3.9±0.52-fold at 24 h ($P<0.05$ vs. control; Fig. 4). The levels of VEGF and bFGF were reduced in the COBRA group vs. the OAβ1-42 group ($P<0.05$; Fig. 4A and B). No significant differences were identified between the OAβ1-42 group and the COBRA treatment group for Ang2 (Fig. 4C).

COBRA reduces capillary-like structure formation. The Matrigel tube formation assay is a quantifiable method of testing the angiogenic properties of compounds on vascular endothelial cells *in vitro*, as it determines the ability of endothelial cells to form capillary-like structures. Cells were cultured on Matrigel-coated wells for 8 h. HUVECs cultured in the cell supernatant of RPE cells, which were exposed to COBRA, exhibited an impaired capacity to form a regular network (due to impaired tube formation ability; Fig. 5C and D). The length of the angiogenic network indicated a significant difference between the COBRA and OAβ1-42 treatment groups (Fig. 5D).

Discussion

Accumulation of drusen in the extracellular compartment between the choroid and the RPE is an early event in the course of AMD (3,4). Aβ is an important constituent of drusen and has been associated with the pathogenesis of AMD (3,4), which has been verified by AMD mice models (25) and RPE cells (7). A previous study also indicated that Aβ may induce inflammation and barrier disruption in RPE cells *in vivo* (11). Additionally, it has been demonstrated that chronic inflammation has a prominent role in the pathogenesis of AMD (26). However, the underlying molecular mechanism is largely unknown. To investigate the dysfunction of Aβ-stimulated RPE cells, the current *in vitro* study was conducted, focusing on angiogenic and inflammatory factors. The present study also demonstrated that OAβ1-42 activates the TLR4, MyD88 and NF-κB signaling pathways, and induced the expression of inflammatory and angiogenic factors in ARPE19 cells. The secretion of cytokines triggered by exposure to OAβ1-42-was significantly reduced when COBRA was applied as a treatment. The capillary-like structures that form as a response to OAβ1-42 were also reduced following COBRA treatment.

The current study verified that OAβ1-42 was the pathological form of Aβ and was responsible for the changes in the mRNA expression levels of cytokines rather than the other forms of Aβ. Additionally, mRNA levels were upregulated, particularly in the OAβ1-42 treatment groups (Fig. 1). The present study also determined mRNA expression levels of other TLRs; however, TLR2 and TLR3 were not increased following OAβ1-42 stimulation (data not shown). Therefore, TLR4 was selected as a target to investigate the pathological function of OAβ1-42 on RPE cells.

Numerous studies have demonstrated the diverse response to TLR4 activation including, the intracellular expression of adaptor protein MyD88, the phosphorylation of IκBα and NF-κB, and elevated mRNA expression of TNF-α, IL-6 and IL-8, along with monocyte chemoattractant protein-1 (MCP-1) (27-29). Qi *et al* (27) indicated that retinal ischemia-reperfusion injury may trigger the TLR4 signaling pathway through MyD88, TNF receptor-associated factor 6 (TRAF6) and NF-κB, leading to the release of mature IL-1β and IL-18. Smith *et al* (28) revealed that infection of macrophages enhanced TNF-α, IL-6 and IL-8 gene expression and protein production in response to the TLR4 ligand (lipopolysaccharide) stimulation. These findings suggest that TLR4-signaling activation, triggered by damage- and pathogen-associated molecular patterns, regulated the release of inflammatory factors in response to various injuries. The current results were consistent with previous studies, and found that OAβ1-42 triggered TLR4 signaling pathway through MyD88 and NF-κB in ARPE19 cells. Additionally, COBRA was able to attenuate the expression of MyD88 and p-NF-κB in OAβ1-42-treated RPE cells. These results suggest the potential function of the TLR4 signaling pathway in the pathological mechanisms of AMD.

The present study indicates that mRNA and protein levels of VEGF, bFGF and Ang2 were upregulated, in the OAβ1-42-treated groups. VEGFA (also termed VEGF165) is considered to be a major angiogenic factor to date (30). bFGF is also important in vascular generation and fibrosis of endothelial cells, RPE cells and membrane formation (31,32). Angiopoietin-2 is also a member of the angiogenic factor family, it facilitates VEGF-induced neovascularization and initiates neovascularization (33). The current results are consistent with those of Yoshida *et al* (7), as stimulation of human RPE cells with Aβ resulted in the altered expression of angiogenic genes, specifically VEGF. The current study provides further evidence that Aβ accumulation affects the balance of angiogenic factors in RPE cells, which may be a key contributor to the development of 'wet' AMD (7).

Cytokines are key drivers of inflammation and their imbalances are often implicated in diseases. Cytokines have gained attention due to their importance in AMD pathophysiology (34). Additionally, IL-6, IL-8 and IL-33 were upregulated in the cell group treated with OAβ1-42. IL-6 is a powerful cytokine that mediates the inflammatory response in a variety of disease states and is implicated in the progression of AMD (34). A previous clinical study determined that the aqueous humor from patients with AMD contains higher concentrations of IL-6 and IL-8 (35). IL-8 is a notable cytokine as its expression may be promoted by IL-1β via direct and indirect mechanisms in RPE cells. Gene microarrays indicated that the mRNA levels of IL-1β and IL-8 were significantly expressed, which is consistent with the present findings (36). In the current study, the concentration of IL-8 increased by 9.17-fold in OAβ1-42-treated RPE cells when compared with the control group. Therefore, IL-8 may account for the observed accumulation of inflammatory cells in the regions of drusen formation in patients with AMD. IL-33 is also important in human inflammatory diseases, and its high level of expression was detected in the inflammatory status of various tissues (37). However, in the present study, IL-33 increased slightly in the COBRA treatment group and it almost recovered to a similar level as the OAβ1-42 only group following 48 h. These results provide support for the hypothesis that OAβ1-42 triggers inflammatory responses in RPE cells and the production of inflammatory factors aiding in the development of AMD. However, inflammatory factors are only one aspect of the progression of AMD.

The present study concluded that COBRA regulates the expression of IL-6, IL-8, IL-33, VEGF, bFGF and Ang2

through suppressing MyD88 and p-NF-κB activation. However, with prolonged exposure, the levels of IL-6, IL-8, IL-33, VEGF and bFGF in the COBRA treatment group approached a similar level as the OAβ1-42 group. Therefore, the release of cytokines in RPE cells induced by Aβ is a complex mechanism, which involves numerous signaling pathways. Previous studies demonstrated that Aβ-induced RPE barrier disruption and expression of inflammation factors was associated with NF-κB, ERK1/2 and p38 MAPK signaling (14,15). The TLR4 signaling pathway is a possible mechanism that is involved with stimulation of inflammatory and angiogenic factors in RPE cells induced by Aβ. This supports the hypothesis that Aβ promotes local inflammation and angiogenic factors near drusen sites and within the surrounding RPE layer that may facilitate the pathogenesis of AMD.

Acknowledgements

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