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# Statins decrease vascular epithelial growth factor expression via down-regulation of receptor for advanced glycation end-products

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## ABSTRACT

**Aims:** Statins, inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase, possess pleiotropic effects that have been extended to modulation of various cellular behaviors. This study aimed to examine whether statins modulate vascular endothelial growth factor A (*VEGF-A*) expression in human retinal pigment epithelium (RPE) cells.

**Main methods:** Human RPE cells (h1RPE7), damaged by hydroquinone (HQ) + advanced glycation endproducts (AGE) in an *in vitro* AMD model, were treated with atorvastatin or lovastatin for 24 h. The expression of *VEGF-A* and receptor for AGE (*RAGE*) was evaluated by real-time RT-PCR. *VEGF-A* secretion was measured by ELISA. To investigate the impact of *RAGE* on *VEGF-A* expression, small interfering RNA (siRNA) for *RAGE* (*siRAGE*) was introduced into h1RPE7 cells and *VEGF-A* expression was measured by real-time RT-PCR. Deletions of *VEGF-A* and *RAGE* promoters were performed and transcriptional activities were measured after the addition of statins to HQ + AGE-damaged RPE cells.

**Key findings:** The mRNA levels of *VEGF-A* and *RAGE* and the levels of VEGF-A in the culture medium were increased by HQ + AGE. Both atorvastatin and lovastatin attenuated HQ + AGE-induced *VEGF-A* and *RAGE* expression. These statins also decreased VEGF-A levels in the culture medium. RNA interference of *RAGE* attenuated the up-regulation of *VEGF-A* in the HQ + AGE treated cells. The deletion analysis demonstrated that these statins attenuated *RAGE* promoter activation in HQ + AGE-damaged RPE cells.

**Significance:** Statins attenuated HQ + AGE-induced VEGF expression by decreasing *RAGE* expression. As VEGF is an important factor in developing wet AMD, statins could decrease the risk of wet-type AMD and be used as preventive medicines.

Keywords: Biological sciences, Ophthalmology, Biochemistry, Cell biology

## 1. Introduction

Age-related macular degeneration (AMD) is an important cause of irreversible visual disorders in elderly patients; the number of AMD patients in 2020 is estimated to be 196 million worldwide, increasing to 288 million by 2040 [1, 2, 3].

The pathogenesis of AMD has traditionally been classified into early and late stages with dry and wet forms. Wet AMD is characterized by choroidal neovascularization and may cause severe visual loss. Several studies have found that vascular endothelial growth factor (VEGF-A) expression in retinal pigment epithelial (RPE) cells is directly related to the pathogenesis/progression of wet AMD, and anti-VEGF drugs are therefore effective for AMD treatment [4, 5, 6]. Anti-VEGF drugs can decrease the progress of this disease, but they exert limited effects on visual acuity. Therefore, development of novel therapeutic agents is required. Statins, commonly used as cholesterol-lowering medications, are inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase and are reported to be useful for decreasing the risk of wet-type AMD [7]. However, the molecular mechanism by which statins reduce the risk of AMD remains unclear. Recently, we reported that the combination of hydroquinone (HQ; benzene-1,4-diol) and advanced glycation endproducts (AGE) increased the expression of *VEGF-A* in RPE cells and that VEGF induced RPE cell proliferation as an autocrine growth factor [8]. In this study, we examined the effects of statins on the expression of *VEGF-A* and on receptor for AGE (*RAGE*) using the HQ + AGE-induced *in vitro* AMD model.

## 2. Materials and methods

### 2.1. Cell culture

Human RPE cells (h1RPE7 cells) [9], were purchased from the European Collection of Cell Cultures (Salisbury, UK) and were grown in Ham's F10 medium

(Gibco<sup>®</sup>, Life Technologies, Carlsbad, CA) containing 20% (v/v) fetal calf serum, 2 mM glutamine (Nacalai tesque, Kyoto, Japan) and 1 µg/mL puromycin (Gibco<sup>®</sup>). For the *in vitro* AMD model, h1RPE7 cells were treated with 300 µg/mL of AGE-bovine serum albumin (BSA) (Calbiochem<sup>®</sup>, Merck KGaA, Darmstadt, Germany) and 20 µM HQ (Wako Pure Chemical Industries, Ltd., Osaka, Japan) as described [8]. ARPE-19 cells were grown in 1:1 mixture of Dulbecco's modified Eagles medium (Gibco<sup>®</sup>) and Ham's F12 medium (Gibco<sup>®</sup>) containing 10% (v/v) fetal calf serum, 100 units/mL penicillin G (Wako), and 100 µg/mL streptomycin (Wako) as described [8].

## 2.2. Induction of VEGF-A and RAGE messenger RNA

The h1RPE7 cells ( $4.0 \times 10^5$  cells/mL in 24-well plates), damaged by 20 µM HQ + 300 µg/mL AGE, were treated with atorvastatin (PubChem CID: 60823) (100 nM, Sigma-Aldrich, St. Louis, MO) or lovastatin (PubChem CID: 53232) (2 µM, Sigma-Aldrich) in Ham's F10 supplemented with 20% lipid-depleted serum (biowest, Nuaille, France) for 24 h. After a 24-h incubation, the h1RPE-7 cells were harvested, and total RNA was prepared as described [8, 10, 11, 12]. The PCR primers corresponding to nucleotides 1131–1151 and 1186–1206 for human *VEGF-A* mRNA (NM\_001025370), 780–799 and 969–988 for human *RAGE* mRNA (NM\_001136), 2708–2727 and 3085–3104 for human *zonula occludens-1* (*ZO-1*) mRNA (NM\_175610), 1051–1069 and 1120–1130 for human  $\alpha$ -smooth muscle actin ( $\alpha$ SMA) (NM\_001613), 415–436 and 502–520 for human *Gremlin* (NM\_013372), 1584–1603 and 1628–1647 for human *transforming growth factor-β1* (*TGFβ1*) (NM\_000660), 1152–1174 and 1565–1586 for human *tyrosinase* (*TYR*) (NM\_000372), 2524–2543 and 2700–2719 for human *MER proto-oncogene tyrosine kinase* (*MERTK*) (NM\_006343), and 420–437 and 492–509 for human  $\beta$ -actin mRNA (NM\_001101) were synthesized by Nihon Gene Research Laboratories (NGRL; Sendai, Japan) as described [8, 10, 11, 12, 13, 14]. Real-time reverse transcription polymerase chain reaction (RT-PCR) was performed using a SYBR<sup>®</sup> Fast qPCR kit (KAPA Biosystems, Wilmington, MA) and the Thermal Cycler Dice<sup>®</sup> Real Time System (Takara Bio Inc., Kusatu, Japan) as described [8, 10, 11, 12, 15]. Target cDNAs were cloned into pBluescript SK(–) plasmid (Stratagene, La Jolla, CA) and sequential 10-fold dilutions from  $10^2$ – $10^7$  copies/µL were prepared. The serial dilutions were run to verify the specificity and to test the sensitivity of the SYBR Green-based real-time RT-PCR. The mRNA expression levels were normalized to the mRNA level of  $\beta$ -actin, which was used to account for differences in the efficiency of reverse transcription between samples.

## 2.3. Measurement of VEGF-A in the culture medium

The h1RPE7 cells ( $4.0 \times 10^5$  cells/mL in 24-well plates), damaged by 20 µM HQ + 300 µg/mL AGE, were treated with atorvastatin (100 nM) or lovastatin (2 µM).

After a 24-h incubation, the cells were given fresh medium and incubated for an additional 3 h at 37 °C. The culture supernatant of the 3-h incubation was collected for measurement of VEGF-A. The concentration of VEGF-A was determined by using a Human VEGF Assay (ELISA; enzyme-linked immunosorbent assay) kit (Immuno-Biological Laboratories Co. Ltd., Fujioka, Japan) according to the manufacturer's instructions.

## 2.4. RNA interference

Small interfering RNA (siRNA) directed against human *RAGE* was synthesized by NGRL. The sense sequence of siRNA for human *RAGE* was 5'-AUCUACAAUUUCUGGCUUCt-3' (corresponding to 466–484 of NM\_001136). The Silencer<sup>®</sup> Select human scrambled siRNA was purchased from Ambion<sup>®</sup> and used as a control. Transfection of siRNAs to h1RPE7 cells was carried out using Lipofectamine<sup>®</sup> RNAiMAX Reagent (Life Technologies) as described [8, 11, 14, 15]. Cells were transfected with 5 pmol per 24-well culture dish ( $4.0 \times 10^5$  cells/mL in 24-well plates).

## 2.5. Construction of reporter plasmid and luciferase assay

The reporter constructs were prepared by inserting the 5'-flanking regions of the human *VEGF-A* gene [16] (−2303 ~ + 50, −605 ~ + 50, −188 ~ + 50, −102 ~ + 50, −78 ~ + 50, −64 ~ + 50, −43 ~ + 50) and human *RAGE* gene (−766 ~ + 29, −253 ~ + 29, −103 ~ + 29) upstream of a firefly luciferase reporter gene in the pGL4.17 [*luc2/Neo*] vector (Promega, Madison, WI). Promoter plasmids were transfected into h1RPE7 cells by using Lipofectamine<sup>®</sup> 3000 (Life Technologies) as described [8]. In brief, h1RPE7 cells were seeded at  $1 \times 10^5$  cells/well in 24-well plates and promoter plasmids were transfected into the cells. After 24 h, each well received fresh medium containing atorvastatin or lovastatin with HQ + AGE and was then incubated for another 24 h. After the treatment, the cells were washed with 1 ml of phosphate-buffered saline, and cell extracts were prepared in extraction buffer (Life Technologies (Tropix<sup>®</sup>): 0.1 M potassium phosphate, pH 7.8/0.2% Triton X-100). To monitor transfection efficiency, pCMV-SPORT-βgal plasmid (Life Technologies) was co-transfected in all experiments at a 1:10 dilution. Luciferase activity was measured using a PicaGene<sup>®</sup> Luciferase assay system (Toyo-ink, Tokyo, Japan) and was normalized by the β-galactosidase activity as described [8, 12, 14, 15, 17, 18].

## 2.6. Data analysis

Results are expressed as mean ± SE. Statistical significance was determined by Student's t-test using GraphPad Prism software (GraphPad Software, La Jolla, CA).

### 3. Results

#### 3.1. VEGF-A gene expression was decreased by statins in the HQ + AGE-treated h1RPE7 cells

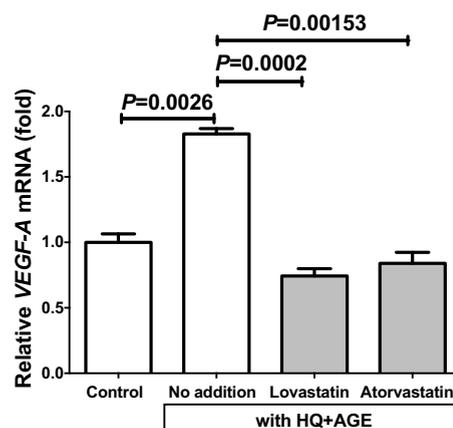
VEGF-A was demonstrated to be an important factor in choroidal angiogenesis and RPE maintenance [19]. Using real-time RT-PCR, we found that the level of *VEGF-A* mRNA in h1RPE7 cells was increased by HQ + AGE ( $P = 0.0026$  vs control). The addition of both lovastatin and atorvastatin attenuated the HQ + AGE-induced *VEGF* expression ( $P = 0.0002$  and  $P = 0.00153$ , respectively) (Fig. 1).

#### 3.2. Statins decreased secreted VEGF-A from the HQ + AGE-treated h1RPE7 cells

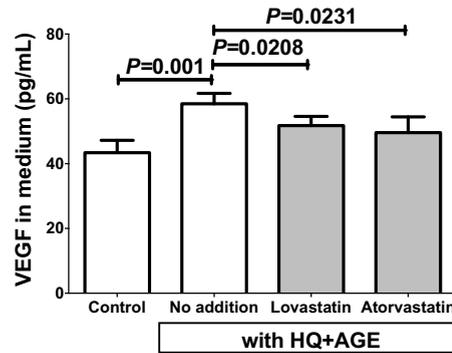
We next measured the concentration of VEGF-A in the h1RPE7 cell culture medium by ELISA and found that it was markedly increased by the treatment with HQ + AGE ( $P = 0.001$ ). Both the additions of lovastatin and atorvastatin attenuated the HQ + AGE-induced VEGF secretion ( $P = 0.0208$  and  $P = 0.0231$ , respectively) (Fig. 2).

#### 3.3. RAGE gene expression was decreased by statins in the HQ + AGE-treated h1RPE7 cells

AGE may influence many signaling pathways, initiated by binding a series of cell surface receptors. The most famous AGE receptor is the multi-ligand receptor for AGE, RAGE [20]. Some other AGE receptors were described as AGE-receptor complexes (AGE-R1/OST-48, AGE-R2/80K-H, AGE-R3/galectin-3) [21, 22] and



**Fig. 1.** Induction of *VEGF-A* expression by the addition of lovastatin or atorvastatin to HQ + AGE-damaged h1RPE7 cells. The h1RPE7 cells were treated with no addition (control), or no addition, lovastatin or atorvastatin in the presence of HQ + AGE for 24 h. *VEGF-A* mRNA was measured by real-time RT-PCR using  $\beta$ -actin as an endogenous control. Data are expressed as mean  $\pm$  SE for each group (n = 4).

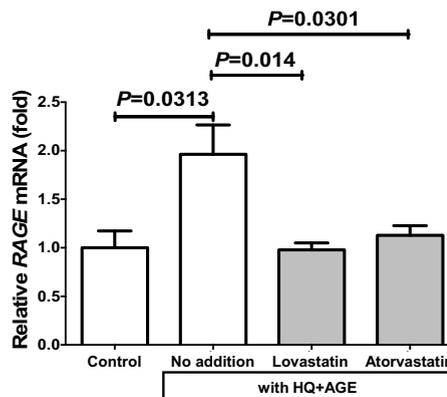


**Fig. 2.** VEGF concentrations in h1RPE7 culture medium. The h1RPE7 human RPE cells were treated with no addition (control), or no addition, lovastatin or atorvastatin in the presence of HQ + AGE for 24 h. Data are expressed as mean  $\pm$  SE for each group (n = 4).

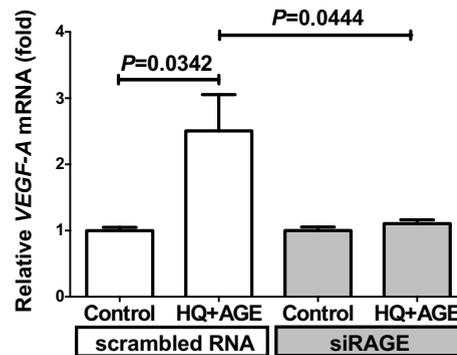
members of the scavenger receptor (SR) family (SR-A [23]; SR-B:CD36 [24, 25]; SR-BI [26]; SR-E:LOX-1 [27]; FEEL-1; FEEL-2 [28]). Using real-time RT-PCR, we found that the level of *RAGE* mRNA in h1RPE7 cells was increased by HQ + AGE ( $P = 0.0313$ ). Both the additions of lovastatin and atorvastatin attenuated the HQ + AGE-induced *RAGE* expression ( $P = 0.014$  and  $P = 0.0301$ , respectively) (Fig. 3).

### 3.4. Downregulation of RAGE attenuated the VEGF increase in the HQ + AGE-treated cells

To determine the mechanism of increased HQ + AGE-stimulated *VEGF* expression, the *RAGE* gene was downregulated by the RNA interference. Knockdown of *RAGE* inhibited the HQ + AGE-induced *VEGF* upregulation ( $P = 0.0444$ ), whereas HQ + AGE treatment induced *VEGF* expression in scrambled



**Fig. 3.** Effects of lovastatin or atorvastatin on expression of *RAGE* in the HQ + AGE-damaged h1RPE7 cells. The h1RPE7 cells were treated with no addition (control), or no addition, lovastatin or atorvastatin in the presence of HQ + AGE for 24 h. *RAGE* mRNA levels were measured by real-time RT-PCR using  $\beta$ -actin as an endogenous control. Data are expressed as mean  $\pm$  SE for each group (n = 4).



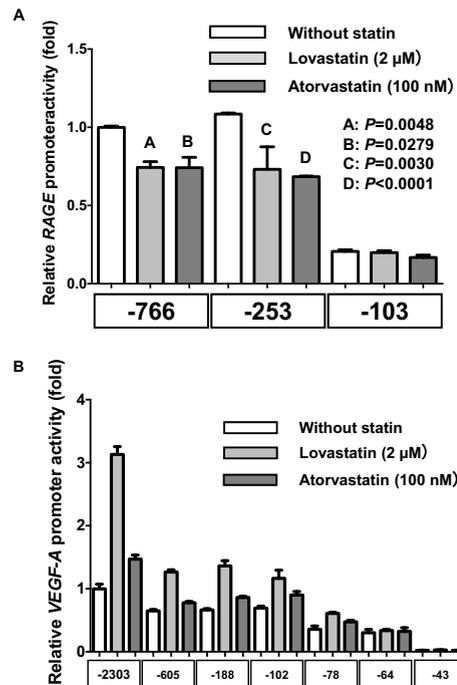
**Fig. 4.** Effect of *siRNA* against *RAGE* on HQ + AGE-induced *VEGF* expression. *SiRNA* of *RAGE* was transfected into h1RPE7 cells and the cells were incubated with HQ + AGE for 24 h. *VEGF-A* mRNA levels were measured by real-time RT-PCR using  $\beta$ -*actin* as an endogenous control. Data are expressed as mean  $\pm$  SE for each group (n = 4).

RNA-introduced cells (Fig. 4). These results indicated that HQ + AGE-induced *VEGF* upregulation was mediated by the AGE-RAGE pathway.

### 3.5. RAGE expression in the statin-treated cells was regulated by the transcriptional level

To identify the type of regulation responsible for *RAGE* and *VEGF-A* gene expression in the HQ + AGE-treated h1RPE7 cells, a 795-bp fragment containing a 766-bp promoter region of the human *RAGE* gene and a 2353-bp fragment containing a 2303-bp promoter region of the human *VEGF-A* gene were fused to the luciferase gene, respectively. Progressive deletions of the *RAGE* and *VEGF-A* promoter genes were performed and the deleted constructs were transfected into h1RPE7 cells. The deletion down to position -253 of the *RAGE* promoter did not significantly alter the expression of the reporter gene, but an additional deletion to nucleotide -103 caused a remarkable decrease of promoter activity in the *RAGE* gene (21% activity of the -766 construct). Both the additions of lovastatin and atorvastatin attenuated the HQ + AGE-induced *RAGE* gene transcription in the -766 and -253 constructs (77% in the -766 and -253 constructs by lovastatin; 77% and 71% in the -766 and -253 constructs by atorvastatin, respectively). However, the inhibitory effects of statins were abolished in the -103 promoter construct (Fig. 5A).

On the other hand, the deletion down to position -102 of *VEGF* promoter did not significantly alter the expression of the reporter gene, maintaining 65%-69% promoter activity, but an additional deletion to nucleotide -78 caused a significant decrease of promoter activity in the *VEGF* gene (36%). The further deletion to -43 abolished the promoter activity (~2%). The addition of lovastatin and atorvastatin did not decrease but rather stimulated, the HQ + AGE-induced *VEGF* gene transcription ( $P < 0.0001$  and  $P = 0.0029$  in the -2303 construct, respectively).



**Fig. 5.** Promoter analyses of *RAGE* (A) and *VEGF-A* (B). The activities of deleted promoters of the human *RAGE* and *VEGF-A* genes are shown. A series of luciferase constructs containing promoter fragments with various 5'-ends were transfected into h1RPE7 cells. After reporter plasmid(s) were introduced into h1RPE7 cells without HQ, AGE, nor statins, cells were incubated for 24 h, and medium was replaced with fresh medium containing atorvastatin or lovastatin with HQ + AGE and was then incubated for another 24 h. The promoter activity was normalized to the activity of co-transfected  $\beta$ -galactosidase plasmid and expressed relative to the activity of  $-766$  without statin in *RAGE* promoter (A) and  $-2303$  without stain in *VEGF* promoter (B). Values are mean  $\pm$  SE for each group ( $n = 4$ ). Statistical significance was expressed by A and B (vs  $-766$  *RAGE* promoter without stain), and C and D (vs  $-253$  *RAGE* promoter without stain).

This stimulatory tendency in the *VEGF* promoter by statin stimulation was observed in all the constructs except for the  $-43$  construct, which lost *VEGF* promoter activity (Fig. 5B)

These results indicate that *RAGE* expression in statin-treated cells was regulated at the transcriptional level, and that the *VEGF* gene in these cells is not regulated by transcription but by a post-transcriptional mechanism.

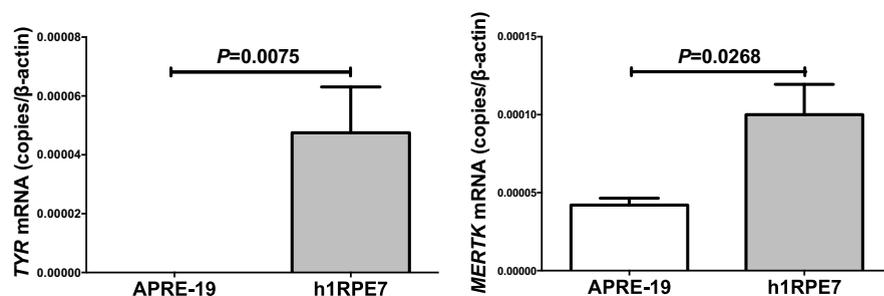
#### 4. Discussion

AMD is a progressive disease and one of the most common causes of severe visual disorders in elderly patients. AMD is multifactorial; cigarette smoking [29, 30], diabetes mellitus [31], obesity [32], and hypertension [33, 34] have all been reported as risk factors for its pathogenesis and progression. However, the

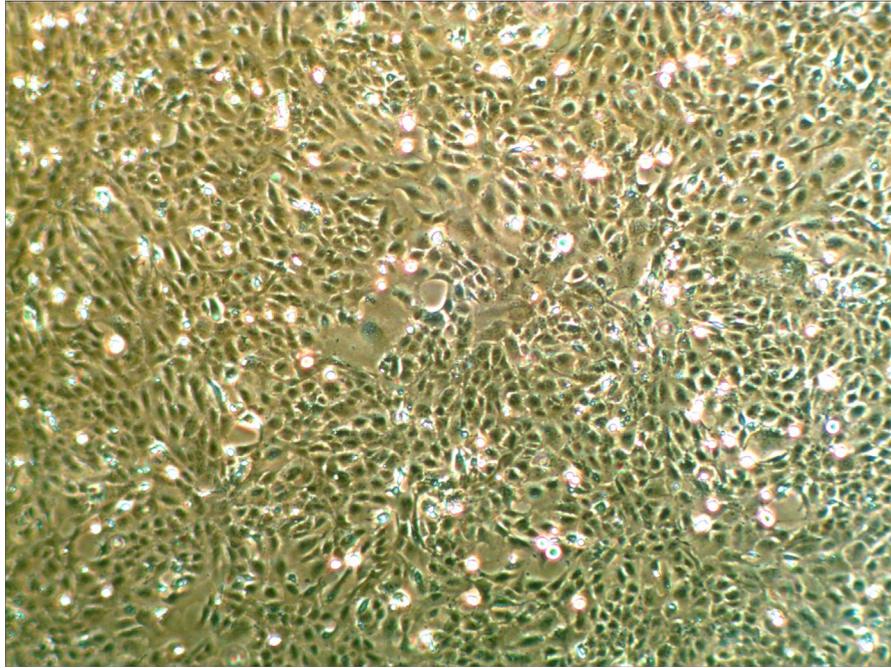
molecular effects of these risk factors remain elusive. Cigarette smoke contains several pro-oxidant compounds among which HQ is the most abundant and important. HQ causes oxidative damage to RPE cells *in vitro* and *in vivo*, and it might play a key role in the pathogenesis of AMD [35, 36, 37, 38]. AGE, which are generated by non-enzymatic reactions between glucose and protein, called the Maillard reaction, are linked to several age-related conditions, including Alzheimer's disease, atherosclerosis, diabetic complications, and AMD [39, 40, 41, 42, 43].

We recently studied the molecular pathogenesis of AMD, and reported that the combined addition of HQ + AGE stimulated VEGF expression and cell proliferation in RPE cells (ARPE-19 and h1RPE7) [8]. Judged by gene expression of *TYR*, encoding a key enzyme for melanin synthesis, and *MERTK*, implicated in phagocytosis of outer segments (Fig. 6), h1RPE7 cells seemed to be more functional than ARPE-19 cells. In addition, h1RPE7 cells grew as contact-inhibited monolayers and exhibited as epithelial morphology (Fig. 7). Thus, we used h1RPE7 cells as RPE cells in this study. The exposure RPE cells (h1RPE7 cells) to HQ + AGE did not change expression of *ZO-1*,  $\alpha$ SMA, *Gremlin*, and *TGF $\beta$ 1* (Fig. 8), indicating that the experimental system seemed to be essentially free from epithelial-mesenchymal transition of retinal pigment epithelial cells. In the present study, we used this model (HQ + AGE in h1RPE7 cells) as an *in vitro* AMD model to seek a new therapy for the wet AMD.

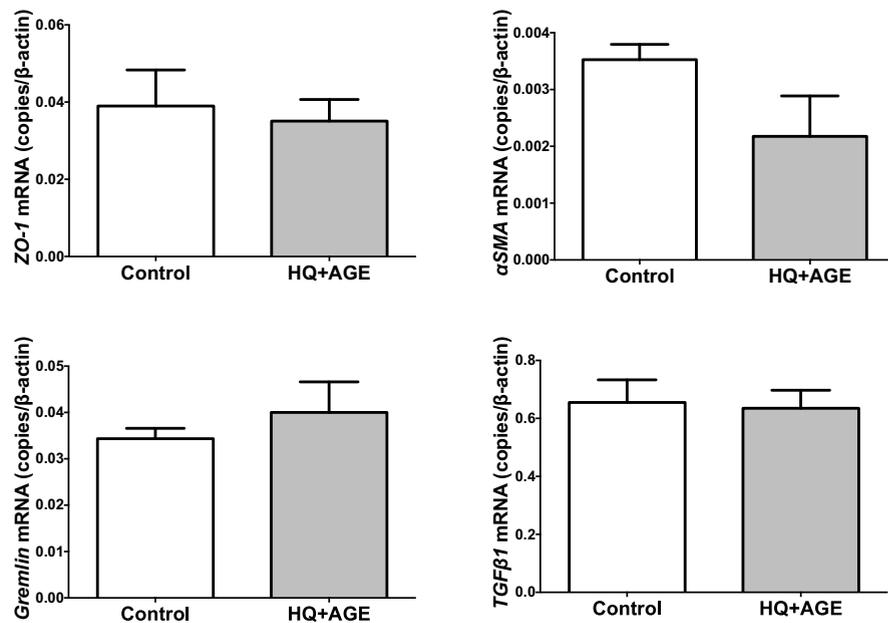
Statins (e.g., atorvastatin and lovastatin), common cholesterol-lowering medications, are inhibitors for 3-hydroxy-3-methylglutaryl coenzyme A reductase, and have recently been reported to be useful for decreasing the risk of wet-type AMD [44]. Statin-induced decrease in serum low-density lipoprotein (LDL) cholesterol might reduce LDL cholesterol deposits in the drusen, which plays a key role in the pathogenesis of AMD [45]. In addition to lipid-lowering effects, statins also exert anti-inflammatory effects through inhibition of the inflammatory pathway [46, 47].



**Fig. 6.** Expression of *TYR* and *MERTK* mRNAs in h1RPE7 and ARPE-19 cells. *TYR* and *MERTK* mRNAs were measured by real-time RT-PCR using  $\beta$ -actin as an endogenous control. Data are expressed as mean  $\pm$  SE for each group (n = 4).



**Fig. 7.** Phase-contrast photomicrograph of h1RPE7 cells. h1RPE7 cells were grown in Ham's F10 medium supplemented with 20% (v/v) fetal calf serum, 2 mM glutamine, and 1  $\mu\text{g}/\text{mL}$  puromycin.



**Fig. 8.** Expression of *ZO-1*,  $\alpha$ *SMA*, *Gremlin*, and *TGF $\beta$ 1* in h1RPE7 human RPE cells treated with no addition or HQ + AGE. Human RPE cells (h1RPE7) were treated with no addition or HQ + AGE for 24 h. *ZO-1*,  $\alpha$ *SMA*, *Gremlin*, and *TGF $\beta$ 1* mRNAs were measured by real-time RT-PCR using  $\beta$ -actin as an endogenous control. Data are expressed as mean  $\pm$  SE for each group (n = 4).  $P = 0.7313$  (*ZO-1*),  $P = 0.1263$  ( $\alpha$ *SMA*),  $P = 0.4494$  (*Gremlin*), and  $P = 0.8480$  (*TGF $\beta$ 1*).

Statins decrease the plasma concentrations of C-reactive protein through attenuation of the amount and function of inflammatory cells [48, 49]. Robinson et al. demonstrated that flavastain downregulated VEGF expression in animal model [50]. Moreover, statins were shown to reduce the intravitreal VEGF levels in patients with idiopathic epiretinal membrane [51], and to reduce drusenoid pigment epithelial detachments and improvement in visual activity [6]. However, the underlying molecular mechanisms by which statins reduce the VEGF expression and secretion remains unclear, especially in RPE cells.

In this study, we examined the effects of statins on the expression of *VEGF-A* and *RAGE* using an *in vitro* AMD model, and showed that both atorvastatin and lovastatin attenuated the HQ + AGE-induced expression of *RAGE* and *VEGF*. Based on RNA interference experiments with *RAGE* mRNA, the HQ + AGE-induced expression of *VEGF-A* mRNA is mediated through the AGE-*RAGE* pathway. Deletion analyses of the *RAGE* promoter indicated that the promoter activity of *RAGE* without statins was maintained to the deletion to -253 and the inhibition of *RAGE* promoter activity by statins remained complete (Fig. 5A). An additional deletion to nucleotide -103 caused a significant decrease in promoter activity without statins and the statin-induced promoter inhibition also disappeared. As region -253 ~ -103 was reported to contain Sp1-binding sites important for *RAGE* gene transcription [52, 53], the Sp1-binding sequences may be important for the HQ + AGE-stimulated *RAGE* transcription as well as the suppression of the transcription by statins. Motoyama et al. reported that the *VEGF* promoter was inhibited by atorvastatin via the induction of sterol regulatory element-binding protein (SREBP) in human smooth muscle cells [54]. In our study, *VEGF* expression seemed to be regulated at the post-transcriptional level not at the transcriptional level (Fig. 5B). Recently, microRNA (miRNA)-mediated regulation has been recognized as a post-transcriptional regulation mechanism; miRNAs appear to regulate the gene expression of more than 60% of the protein-coding genes of the human genome [55]. Hao et al. recently showed that miR-146a is highly related to the expression of *VEGF-A* in RPE cells [56]. Although the mechanism used by statins to reduce the HQ + AGE-induced expression of *VEGF-A* mRNA is elusive, statins could reduce the risk of wet AMD by decreasing the expression of *RAGE* and *VEGF-A* and therefore may be potential new drugs for preventing and treating wet AMD.

## 5. Conclusions

Statins attenuated HQ + AGE-induced *VEGF* expression by decreasing *RAGE* expression, which was attenuated at the transcriptional level. By decreasing the levels of *VEGF-A*, a key factor in the pathogenesis of wet AMD, statins may potentially be used for preventing and treating this disease.

## Declarations

### Author contribution statement

Hiroki Tsujinaka, Asako Itaya-Hironaka, Shin Takasawa: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Akiyo Yamauchi, Sumiyo Sakuramoto-Tsuchida, Ryogo Shobatake, Mai Makino, Naonori Masuda, Hiromasa Hirai, Nahoko Ogata: Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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### Competing interest statement

The authors declare no conflict of interest.

### Additional information

No additional information is available for this paper.

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