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# Human retinal pigment epithelial cell proliferation by the combined stimulation of hydroquinone and advanced glycation end-products via up-regulation of VEGF gene



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## ABSTRACT

Although recent research showed that advanced glycation endproduct (AGE) and hydroquinone (HQ) are related to the pathogenesis of age-related macular degeneration (AMD), the mechanism how AGE and HQ induce or accelerate AMD remains elusive. In the present study, we examined the effects of AGE and HQ on changes of human retinal pigment epithelial (RPE) cell numbers and found that the viable cell numbers were markedly reduced by HQ by apoptosis and that AGE prevented the decreases of HQ-treated cell numbers by increased replicative DNA synthesis of RPE cells without changing apoptosis. Real-time RT-PCR revealed that *vascular endothelial growth factor (VEGF)-A* mRNA was increased by HQ treatment and the addition of HQ+AGE resulted in a further increment. The increase of VEGF secretion was confirmed by ELISA, and inhibition of VEGF signaling by chemical inhibitors and small interfering RNA decreased the HQ+AGE-induced increases in RPE cell numbers. The deletion analysis demonstrated that –102 to –43 region was essential for the *VEGF-A* promoter activation. Site-directed mutations of specificity protein 1 (SP1) binding sequences in the *VEGF-A* promoter and RNA interference of SP1 revealed that SP1 is an essential transcription factor for *VEGF-A* expression. These results indicate that HQ induces RPE cell apoptosis, leading to dry AMD, and suggest that AGE stimulation in addition to HQ enhances *VEGF-A* transcription via the AGE-receptor for AGE pathway in HQ-damaged cells. As a result, the secreted VEGF acts as an autocrine/paracrine growth factor for RPE and/or adjacent vascular cells, causing wet AMD.

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## 1. Introduction

Age-related macular degeneration (AMD) is the major cause of irreversible blindness in elderly patients in the world and the numbers of people with AMD in 2020 are estimated to be 196 million, increasing to 288 million in 2040 in the world [1–3]. In the

pathogenesis of AMD, this disease has been traditionally classified into early and late stages with its dry and wet forms. The dry AMD is defined as presence of drusen, retinal pigment epithelial (RPE) cell changes, and progressive destruction of RPE cells and wet AMD, usually associated with greater visual loss, is characterized by choroidal neovascularization which is led by some angiogenic cytokines such as vascular endothelial growth factor (VEGF). Although the pathophysiology of AMD is not yet fully understood, this multifactorial progressive disease likely arises from a complex interaction of genetic and environmental risk factors [4–6]. Cigarette smoking and aging are one of the most significant risk factors in the onset and severity of both dry and wet AMD. Cigarette smoke contains a large number of pro-oxidant compounds among which benzene-1,4-diol (hydroquinone: HQ) is the most abundant and important. HQ cause oxidative damage to RPE cells in vitro and vivo and it might play a key role in the pathogenesis of AMD [7–10]. Advanced glycation endproduct (AGE), which are

**Abbreviations:** AGE, advanced glycation endproduct; AMD, age-related macular degeneration; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; HQ, hydroquinone; IdU, 5'-Iodo-2'-deoxyuridine; RAGE, receptor for advanced glycation endproduct; RPE, retinal pigment epithelial; RT-PCR, reverse transcription polymerase chain reaction; siRNA, small interfering RNA; SP1, specificity protein 1; SR, scavenger receptor; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; VEGF, vascular endothelial growth factor; WST-8, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-dinitrophenyl)-2H-tetrazolium monosodium salt

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generated by non-enzymatic reactions between glucose and protein called Maillard reaction, are linked to several age-related diseases such as Alzheimer's disease, atherosclerosis, diabetic complications and AMD [11–15]. Hahn et al. recently demonstrated that the incidence of AMD was significantly higher in the diabetes mellitus patients with nonproliferative diabetic retinopathy [16]. While AGEs have been shown to stimulate inflammation and played the important role in other diseases, whether it plays a similar role in AMD is not known. In the progression of the AMD, VEGF is shown to be a most important cytokine [17]. Aberrant VEGF-A expression in RPE cells has been demonstrated to promote the progression of the choroidal neovascularization associated with wet AMD. However, little is known about the molecular regulation of VEGF-A in RPE cells.

In the present study, we investigated effects of AGEs on cell proliferation and VEGF-A expression in the HQ-damaged human RPE cells. We also showed HQ+AGEs induced VEGF-A transcription via specificity protein 1 (SP1) in human RPE cells.

## 2. Material and methods

### 2.1. Cell culture

Two human RPE cell lines, ARPE-19 cells [18] and h1RPE7 cells [19], were evaluated separately. ARPE-19 cells were grown in 1:1 mixture of Dulbecco's modified Eagles medium (Gibco<sup>®</sup>, Life Technologies, Carlsbad, CA) and Ham's F12 medium (Gibco<sup>®</sup>) containing 10% (v/v) fetal calf serum (FCS), 100 units/ml penicillin G (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and 100 µg/ml streptomycin (Wako). h1RPE7 cells were purchased from European Collection of Cell Culture (Salisbury, UK) and were grown in Ham's F10 medium (Gibco<sup>®</sup>) containing 20% (v/v) FCS, 2 mM glutamine (Nacalai tesque, Kyoto, Japan) and 1 µg/ml puromycin (Gibco<sup>®</sup>). For the stimulation experiments, ARPE-19 cells were treated with 300 µg/ml AGE-bovine serum albumin (BSA) (Calbiochem<sup>®</sup>, Merck KGaA, Darmstadt, Germany), and/or 20 µM HQ (Wako). h1RPE7 cells were treated with 300 µg/ml AGE-BSA and/or 40 µM HQ.

### 2.2. Measurement of viable cell numbers by tetrazolium salt cleavage

ARPE-19 and h1RPE7 cells ( $0.5\text{--}2.0 \times 10^4$  cells/100 µL in 96-well plate) were incubated with the addition of AGEs and/or HQ for 24 h. After the treatment, the viable cell numbers were determined by a Cell Counting kit-8 (Dojindo Laboratories, Mashikimachi, Japan) according to the manufacture's instructions as described [20–22]. Briefly, WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazoliummonosodium salt) solution was added to cells in 96-well plates, and the cells were incubated at 37 °C for 1–2 h. The optical density of each well was read at 450 nm (reference wave length at 650 nm) using a Sunrise<sup>™</sup> microplate reader (Tecan, Männedorf, Switzerland).

To investigate effects of VEGF-A on cell growth in RPE cells, ARPE-19 cells ( $2.0 \times 10^4$  cells/100 µL in 96-well plate) were incubated with AGEs, HQ, in the presence of VEGF-A/VEGF-A receptor inhibitors (10 µg/ml sulochrin (Sigma-Aldrich, St. Louis, MO) [23], 3 nM Ki8751 (Calbiochem<sup>®</sup>) [24] or 50 nM CBO-P11 (Calbiochem<sup>®</sup>) [25]), or siRNA against VEGF or receptor for AGE (RAGE) for 24 h. After the treatment, the viable cell numbers were determined by a Cell Counting kit-8 (Dojindo) according to the manufacture's instructions.

### 2.3. Measurement of apoptosis

ARPE-19 cells ( $0.5 \times 10^4$  cells/100 µL in 96-well plate) were treated with AGEs and/or HQ for 12 h. After the treatment, apoptosis was detected by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method using Apoptosis Screening Kit (Wako) as described [21,22,26,27]. The optical density of each well was read at 490 nm (reference wave length at 650 nm).

### 2.4. Measurement of replicative DNA synthesis

5'-Indo-2'-deoxyuridine (IdU) solution was added in the culture medium of ARPE-19 cells ( $2.0 \times 10^4$  cells/100 µL in 96-well plate), and after a 12 h incubation in the presence of AGEs and/or HQ, IdU incorporation was measured using a DNA-IdU Labeling and Detection kit (Takara Bio Inc., Otsu, Japan) as described [26,27]. The optical density of each well was read at 490 nm (reference wave length at 650 nm).

### 2.5. Induction of VEGF-A messenger RNA

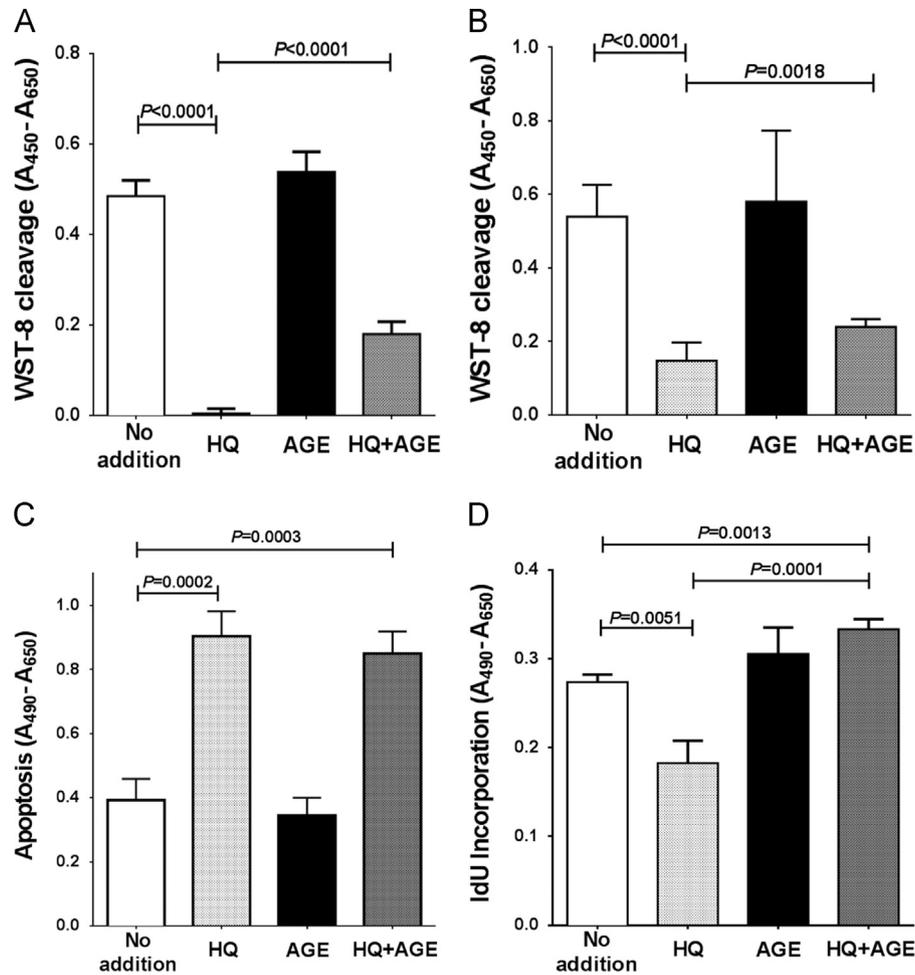
After a 12 h incubation with AGEs and/or HQ, ARPE-19 cells were harvested, and total RNA was prepared as described [21,22,28]. The PCR primers corresponding to nucleotides 1131–1151 and 1186–1206 for human VEGF-A mRNA (NM\_001025370), 305–329 and 374–395 for human specificity protein 1 (SP1) mRNA (NM\_138473), 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 [20–22,26–31]. Real-time reverse transcription polymerase chain reaction (RT-PCR) was performed using SYBR<sup>®</sup> Fast qPCR kit (KAPA Biosystems, Wilmington, MA) and Thermal Cycle Dice<sup>®</sup> Real Time System (Takara Bio Inc.) as described [20–22,28,30]. Target cDNAs were cloned into pBluescript SK(-) plasmid (Stratagene, La Jolla, CA) and sequential 10-fold dilutions from  $10^2\text{--}10^7$  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 difference in the efficiency of reverse transcription between samples.

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

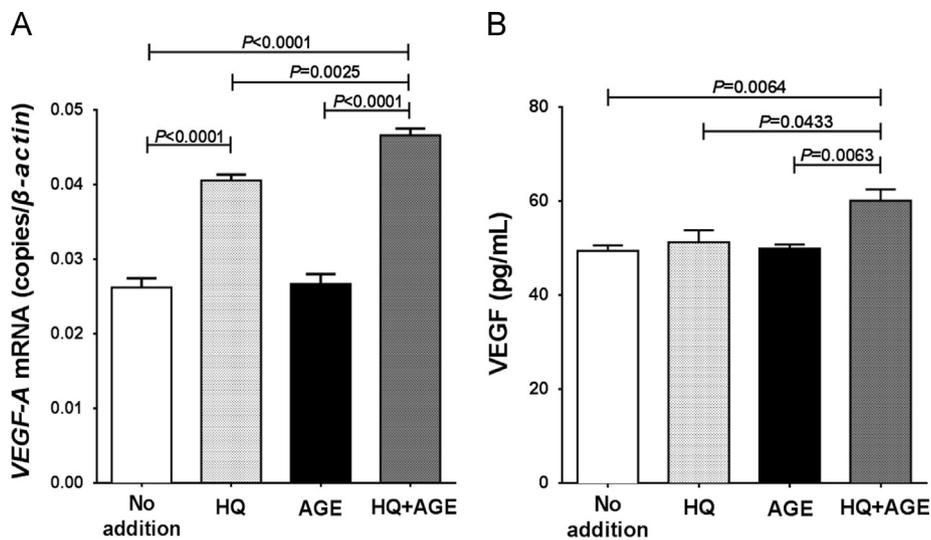
ARPE-19 cells ( $4.0 \times 10^4$  cells/1 mL in 24-well plate) were incubated with the addition of AGEs and/or HQ for 12 h, and then the medium was changed to a fresh medium. After additional 3 h incubation with fresh medium, culture medium was collected and the concentration of VEGF-A was measured by using a Human VEGF Quantikine ELISA (enzyme-linked immunosorbent assay) kit (R&D Systems Inc., Minneapolis, MN) according to the instructions of supplier.

### 2.7. Construction of reporter plasmid and luciferase assay

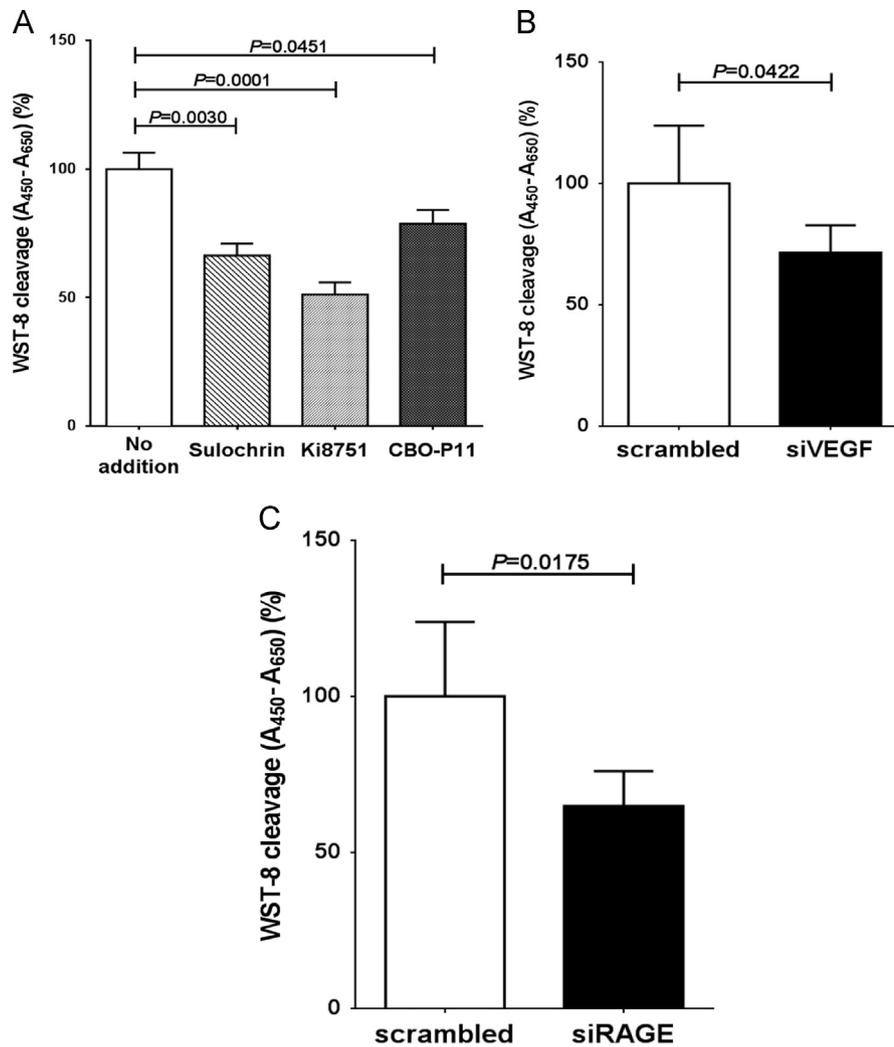
The reporter constructs were prepared by inserting the 5'-flanking regions of human VEGF-A gene [32] (–2303 to +50, –1778 to +50, –1293 to +50, –826 to +50, –605 to +50, –358 to +50, –188 to +50, –102 to +50, –78 to +50, –64 to +50, –43 to +50) upstream of a firefly luciferase reporter gene in pGL4.17[luc2/neo] vector (Promega, Madison, WI). Mutants of potential binding sites for SP1 and their adjacent sequence (SP1 M1~7) were constructed on pGL4.17[luc2/neo] vector containing the VEGF promoter by PCR. Promoter plasmids were transfected into ARPE-19 and h1RPE-7 cells by using Lipofectamine<sup>™</sup> 2000 (Life Technologies) as described [28]. In brief, ARPE-19 and h1RPE7 cells were seeded at  $1 \times 10^5$  cells per well in 24-well plate and



**Fig. 1.** AGE partially prevented the loss of HQ-treated cell numbers by increases of replicative DNA synthesis. ARPE-19 cells (A) and hRPE7 cells (B) were incubated for 24 h with HQ and/or AGE and viable cell numbers were measured by WST-8 assay. Data are expressed as means  $\pm$  SEM for each group ( $n=4-6$ ). (C) Apoptosis of ARPE-19 cells treated with HQ and/or AGE. ARPE-19 cells were incubated with HQ and/or AGEs for 12 h and apoptosis was quantified by TUNEL method. Data are expressed as means  $\pm$  SEM for each group ( $n=4$ ). (D) Replicative DNA synthesis of ARPE-19 cells incubated with HQ and/or AGEs. ARPE-19 cells were treated with HQ and/or AGEs for 12 h and replicative DNA synthesis were measured by IdU incorporation. Data are expressed as means  $\pm$  SEM for each group ( $n=7$ ).



**Fig. 2.** Induction of VEGF-A expression by the addition of HQ and/or AGE. (A) Expression of VEGF-A mRNA in ARPE-19 cells. ARPE-19 cells were treated with no addition, HQ, AGEs or combinations for 12 h. The level of VEGF-A mRNA was measured by real-time RT-PCR using  $\beta$ -actin as an endogenous control. Data are expressed as means  $\pm$  SEM for each group ( $n=4$ ). (B) Concentrations of VEGF in the ARPE-19 culture medium were measured by ELISA. ARPE-19 cells were treated with no addition, HQ, AGEs or combinations for 12 h. Data are expressed as means  $\pm$  SEM for each group ( $n=4$ ).



**Fig. 3.** Inhibition of ARPE-19 cell proliferation by inhibition of VEGF signaling. (A) Effects of the VEGF inhibitors on cell proliferation. ARPE-19 were incubated with HQ+AGEs and three VEGF-A inhibitors, 10  $\mu$ g/mL Sulochrin, 3 nM Ki8751 or 50 nM CBO-P11 for 12 h. After the treatment, cellular proliferation was measured by WST-8 assay. Data are exposed as means  $\pm$  SEM for each group ( $n=6$ ). (B) Effect of siRNA against *VEGF-A* on cell proliferation. SiRNA of *VEGF-A* was transfected into ARPE-19 cells and the cells were incubated with HQ+AGEs for 12 h. Cellular proliferation was measured by WST-8 assay. Data are expressed as means  $\pm$  SEM for each group ( $n=5$ ). (C) Effect of siRNA against *RAGE* on HQ+AGE-induced ARPE-19 cell proliferation. SiRNA of *RAGE* was transfected into ARPE-19 cells and the cells were incubated with HQ+AGEs for 12 h. Cellular proliferation was measured by WST-8 assay. Data are exposed as means  $\pm$  SEM for each group ( $n=5$ ).

promoter plasmids were transfected into the cells. After 12 h from transfection, the medium of each well was replaced with fresh medium containing HQ and/or AGEs and incubated for another 12 h. After the treatment, cells were washed with 1 ml of phosphate buffered saline, and cell extracts were prepared in extraction buffer (Life Technologies: 0.1 M potassium phosphate, pH 8.8/0.2% Triton X-100). To monitor transfection efficiency, pCMV-SPORT- $\beta$ gal plasmid (Life Technologies) was co-transfected in all experiments at a 1:10 dilution. Luciferase activity was measured using a PicaGene Luciferase assay system (Toyo-ink, Tokyo, Japan) and was normalized by the  $\beta$ -galactosidase activity as described [28,29,33–36].

### 2.8. RNA interference

RNA interference against human *SP1* was performed using Silencer<sup>®</sup> Select predesigned small interfering RNAs (siRNAs) (Ambion<sup>®</sup>, Life Technologies). The sense sequence of siRNA for human *SP1* was 5'-GCAACAUGGGAAUUAUGAAtt-3'. SiRNAs directed against human *VEGF-A* and *RAGE* were synthesized by NGRL.

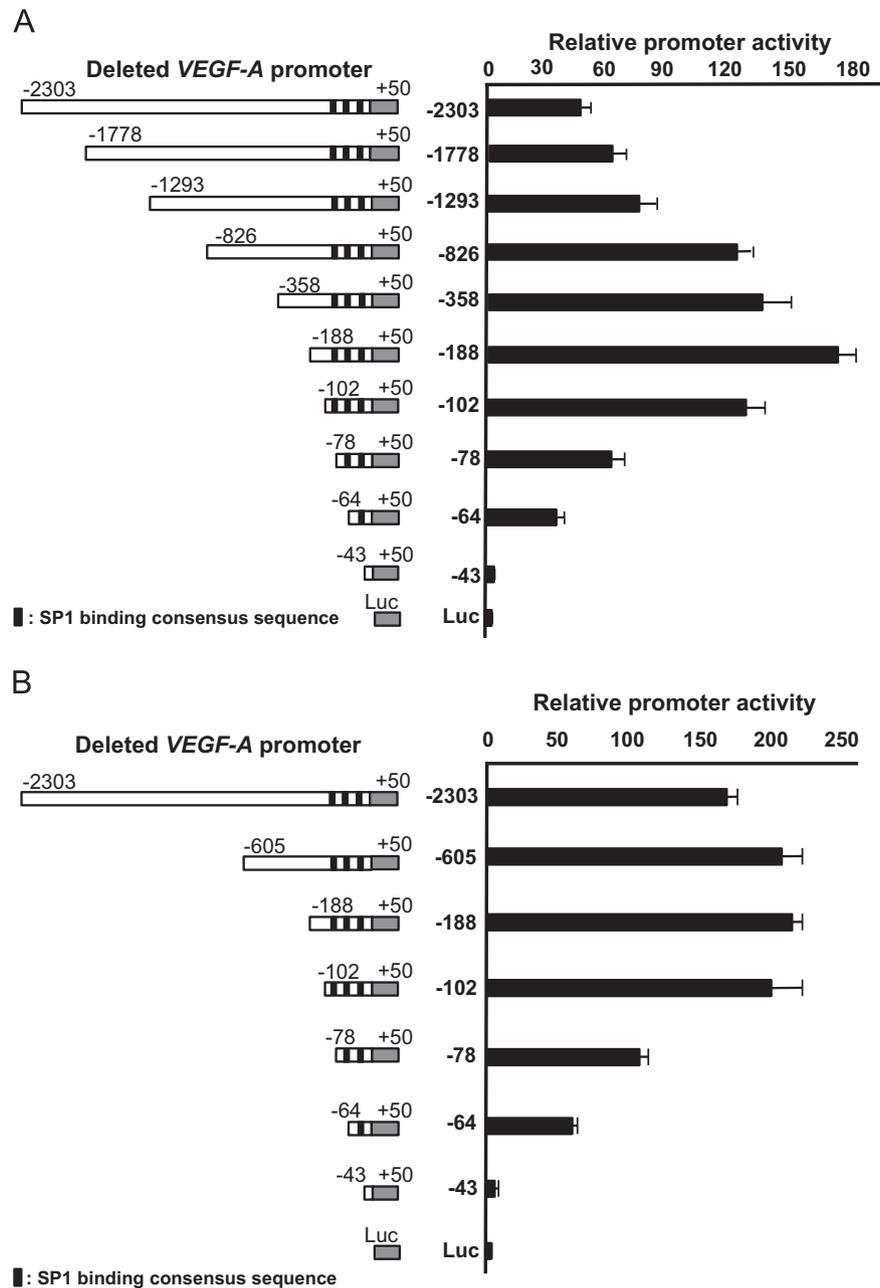
The sense sequence of siRNA for human *VEGF-A* and *RAGE* were 5'-GGAGUACCCUGAUGAGAUCtt-3' and 5'-AUCUACA AUUUCUGGCUUCtt-3'. The Silencer<sup>®</sup> Select human scrambled siRNA was purchased from Ambion<sup>®</sup> and used as a control. Transfection of siRNAs to ARPE-19 cells was carried out using Lipofectamine<sup>®</sup> RNAiMAX Reagent (Life Technologies) as described [22,36]. Cells were transfected with 5 pmol/24-well culture dish ( $1.5 \times 10^5$  cells/mL) for real-time RT-PCR and 1 pmol/96-well culture dish ( $0.5 \times 10^4$  cells/100  $\mu$ L) for WST-8 assay.

### 2.9. Date analysis

Results are expressed as mean  $\pm$  SEM. Statistical significance was determined by Student's *t*-test using Graph Pad Prism (GraphPad Software, La Jolla, CA).

## 3. Results

### 3.1. AGE partially prevented the loss of HQ-treated cell numbers by



**Fig. 4.** Localization of essential region for VEGF-A transcription. The promoter activity on deleted promoter of human VEGF-A gene was shown. A series of luciferase constructs containing promoter fragments with various 5'-ends were transfected into (A) ARPE-19 and (B) h1RPE7 cells. The promoter activity was normalized and expressed relative to the activity of co-transfected  $\beta$ -galactosidase plasmid and was expressed relative to the activity of promoterless pGL4.17[luc2/neo]. Values are means  $\pm$  SEM for each group ( $n=3-4$ ). Possible binding sites for SP1 in the promoter region were black labeled in left panel.

#### increases of replicative DNA synthesis

To evaluate direct effects of AGE and/or HQ on RPE cell proliferation/cell death, ARPE-19 and h1RPE7 cells were exposed to AGE, HQ, or HQ+AGE for 24 h. After the treatment, the viable cell numbers were determined by WST-8 assay. As shown in Fig. 1A and B, the viable cell numbers of both ARPE-19 and h1RPE7 were markedly reduced by HQ treatment ( $P < 0.0001$ ). The addition of AGE did not show changes of viable cell numbers both in ARPE-19 and h1RPE7 cells (No addition vs AGE,  $P=0.3611$  in ARPE19, and  $P=0.6452$  in h1RPE7). On the other hand, the combined addition of HQ and AGE showed increased cell numbers against the addition of HQ alone ( $P < 0.0001$  in ARPE-19 and  $P=0.0018$  in hRPE7).

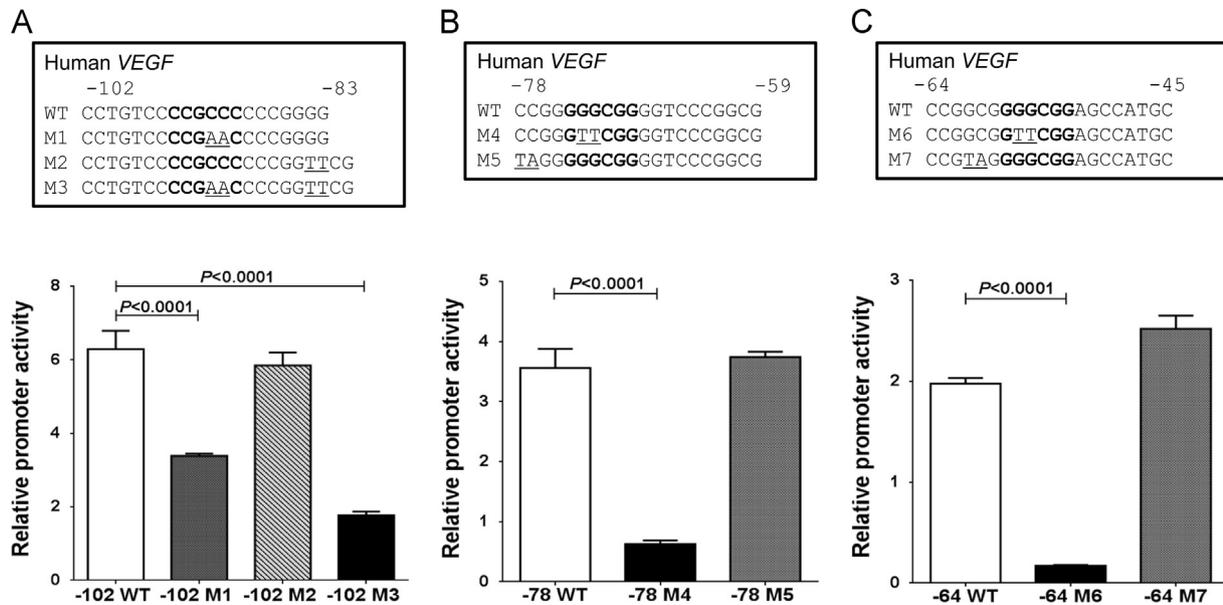
To determine whether HQ increases apoptosis, we measured apoptosis of ARPE-19 cells by TUNEL method. As shown in Fig. 1C,

HQ significantly increased apoptosis ( $P=0.0002$ ). AGE alone did not change apoptosis and the addition of HQ+AGE did not prevent the HQ-induced apoptosis, suggesting that AGE stimulates proliferation of HQ-treated RPE cells.

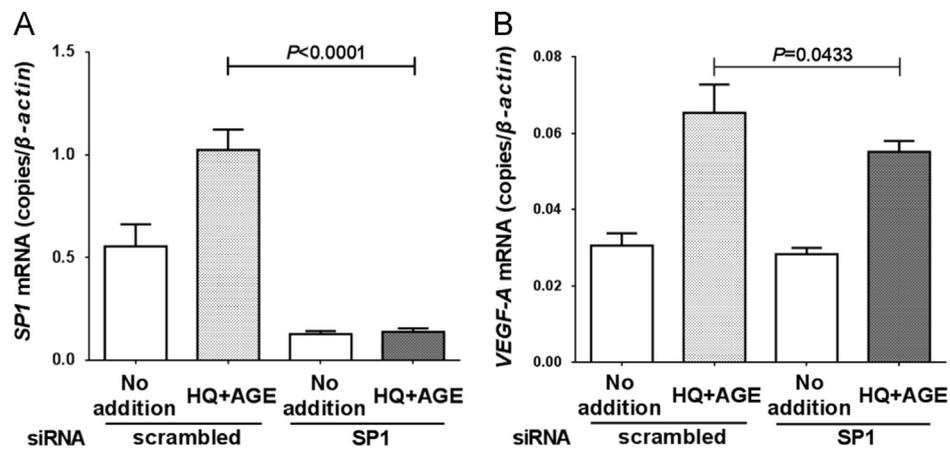
We then measured replicative DNA synthesis of ARPE-19 cells by IdU incorporation. As shown in Fig. 1D, replicative DNA synthesis was increased by the addition of AGE in HQ-treated cells (No addition vs HQ+AGE,  $P=0.0013$ ; HQ vs HQ+AGE,  $P=0.0001$ ), indicating that AGE increases cell number by activating replicative DNA synthesis in HQ-treated cells.

#### 3.2. HQ+AGE increased VEGF-A mRNA and secreted VEGF in the medium

VEGF-A has been shown to be an important regulator of



**Fig. 5.** Effects of site-directed mutations on *VEGF-A* promoter activity. (A) Three site-directed mutations from  $-102$  to  $-83$ , *VEGF* M1–3 are indicated. *VEGF* M1 and M3 markedly decreased the promoter activity induced by HQ+AGEs. (B) Two site-directed mutations from  $-78$  to  $-59$ , *VEGF* M4 and M5 are indicated. *VEGF* M4 markedly decreased the promoter activity. (C) Two site-directed mutations from  $-64$  to  $-45$ , *VEGF* M6 and M7 are indicated. *VEGF* M6 markedly decreased the promoter activity. GC box sequences, which are possible binding sites for SP1 in the *VEGF-A* promoter, were shown by bold, and the mutation sites were underlined. Values are means  $\pm$  SEM for each group ( $n=3$ ).



**Fig. 6.** Effect of *SP1* knockdown on *VEGF-A* mRNA expression in ARPE-19 cells. After siRNA introduction, ARPE-19 cells were exposed with HQ+AGE. The mRNA levels of (A) *SP1* and (B) *VEGF-A* were measured by real-time RT-PCR. Data are expressed as means  $\pm$  SEM for each group ( $n=4$ ).

pathological angiogenesis and RPE proliferation [37]. We analyzed the mRNA levels of *VEGF-A* by real-time RT-PCR and found that the level of *VEGF-A* mRNA was increased by HQ treatment ( $P < 0.0001$  vs no addition) and the addition of HQ+AGE resulted in a further increment of *VEGF-A* mRNA ( $P=0.0025$  vs HQ) (Fig. 2A). We next measured the concentration of *VEGF-A* in the ARPE-19 cell culture medium by ELISA and found that the concentration of *VEGF-A* was significantly increased by the treatment of HQ+AGE ( $P=0.0064$  vs no addition,  $P=0.0433$  vs HQ,  $P=0.0063$  vs AGE) (Fig. 2B).

### 3.3. Inhibition of the *VEGF-A* signaling decreased the cell proliferation

In order to know the mechanism of HQ+AGE-stimulated RPE cell proliferation, we first tested effects of VEGF/VEGF receptor inhibitors such as sulochrin, Ki8751, and CBO-P11 on HQ+AGE-induced cell proliferation. As shown in Fig. 3A, the WST-8 cleavage of ARPE-19 cells treated with HQ+AGE was significantly reduced by sulochrin, Ki8751, and CBO-P11 (Fig. 3A). RNA interference of *VEGF-A* also inhibited the HQ+AGE-induced cell proliferation (Fig. 3B) ( $P=0.0422$ ). These results indicated that HQ+AGE-

induced RPE cell proliferation was mediated by *VEGF-A* expression.

Formation of AGEs leads to activation of variable signaling pathways, initiating by a series of cell surface receptors. The most studied AGE-receptor is the multi-ligand receptor for AGE, RAGE [38]. Several other AGE-receptors were also identified as AGE-receptor complex (AGE-R1/OST-48, AGE-R2/80K-H, AGE-R3/galectin-3) [39,40] and some members of the scavenger receptor (SR) family (SR-A [41]; SR-B: CD36 [42,43]; SR-BI [44]; SR-E: LOX-1 [45]; FEEL-1; FEEL-2 [46]). In order to identify which AGE-receptor was used in the HQ+AGE-stimulated RPE cell proliferation, we introduced siRNA against *RAGE* into ARPE-19 cells and treated cells with HQ+AGE, and found that the viable cell numbers of siRAGE-introduced cells were significantly decreased than those of control cells (Fig. 3C), indicating that AGE signal is mediated mainly by RAGE in HQ-treated RPE cells.

### 3.4. Localization of the *VEGF-A* promoter region

To identify the region necessary for the induction of the *VEGF-A*

gene in the HQ+AGE-treated RPE cells, a 2353 bp fragment containing 2303 bp of the promoter region of the human *VEGF-A* was fused to the luciferase gene. Progressive deletions of the *VEGF-A* promoter gene were performed and the deleted constructs were transfected into ARPE-19 and hRPE7 cells. As shown in Fig. 4, the deletion down to position  $-102$  did not alter significantly the expression of the reporter gene, but an additional deletion to nucleotide  $-78$  caused a remarkable decrease of promoter activity. Furthermore, deletions to nucleotide  $-64$  and to nucleotide  $-43$  caused further decreases of promoter activity, indicating that the regions from  $-102$  to  $-78$ ,  $-78$  to  $-64$  and  $-64$  to  $-43$  contain essential *cis*-elements for the *VEGF-A* promoter activities. A computer-aided search for sequences similar to known *cis*-acting element revealed that each region has a GC box sequence that is possible binding sites for SP1.

### 3.5. SP1 is a key factor for *VEGF-A* transcription

To map out the *cis*-element of *VEGF* promoter that is responsible for *VEGF* transcription, site-directed mutagenesis of the possible transcription factor binding sites was conducted within the luciferase construct of “ $-102$ ”, “ $-78$ ”, and “ $-64$ ”. *VEGF* M1~7, which were introduced mutations in the SP1 binding motif (s) and its nearby sequence, were constructed and introduced them into ARPE-19 cells (Fig. 5). *VEGF* M1 and M3 showed significant reductions in promoter activities ( $P < 0.0001$ ), while *VEGF* M2 showed almost the same promoter activity as that of  $-102$  wild-type (WT) construct. *VEGF* M4 showed significant reductions in promoter activities ( $P < 0.0001$ ), while *VEGF* M5 showed almost the same promoter activity as that of  $-78$  WT. Moreover, *VEGF* M6 showed significant reductions in promoter activities ( $P < 0.0001$ ), while *VEGF* M7 showed almost the same promoter activity as that of  $-64$  WT. These results strongly suggest that SP1 binding to the GC box sequences is very important for *VEGF-A* transcription in RPE cells.

To investigate significance of SP1 transcription factor for *VEGF-A* expression in RPE cells, we used RNA interference of SP1 to identify whether SP1 is essential for the transcription of *VEGF-A* gene. As shown in Fig. 6, the introduction of SP1 siRNA into the ARPE-19 cells reduced not only SP1 mRNA itself but also HQ+AGE-induced *VEGF-A* mRNA ( $P = 0.0433$ ). These results provided evidence that SP1 binds to the GC boxes in the *VEGF-A* promoter to up-regulate *VEGF-A* transcription in RPE cells in response to HQ+AGE treatment.

## 4. Discussion

AMD is a progressive disease and one of the great causes of severe vision loss in the elderly patients. AMD has also been recognized as a multifactorial disease, for example, cigarette smoking [47,48], diabetes mellitus [6], obesity [49], and hypertension [50,51] have been reported as risk factors of AMD pathogenesis and progression. This disease has been also traditionally classified into early and late stages with its dry and wet forms. The dry form AMD was defined as progressive destruction of retinal pigment epithelial cells. While, wet form AMD is characterized by choroidal neovascularization, which is led by some angiogenic cytokines such as VEGF. Despite of intensive researches, what determine AMD subtypes (dry or wet type) has been obscured.

In the present study, we found that the viable RPE cell numbers were markedly reduced by HQ treatment, and the combined addition of HQ+AGE increased cell numbers against HQ-treated cells. TUNEL assay demonstrated that HQ increased apoptosis of RPE cells. On the other hand, replicative DNA synthesis was stimulated by the addition of AGE in HQ-treated RPE cells. Real-time

RT-PCR revealed that the level of *VEGF* mRNA was increased by HQ treatment and the addition of HQ+AGE resulted in a further increment of *VEGF-A* mRNA. ELISA certified that the VEGF secretion from RPE cells to the culture medium was increased by the addition of HQ+AGE. As VEGF has already demonstrated to proliferate RPE cells and cause the choroidal neovascularization [52,53], it is quite possible that RPE cell proliferation and choroidal vessel induction are caused by HQ+AGE-induced VEGF expression from HQ-damaged RPE cells (Figs. 2 and 3). As how AGE upregulates *VEGF* expression in RPE cells was unclear, we performed introduction of siRAGE into RPE cells and found siRAGE significantly inhibited the proliferation of HQ+AGE-treated RPE cells, indicating an essential role of the AGE-RAGE pathway in *VEGF* induction in HQ+AGE-treated RPE cells. Although regulation of *VEGF-A* gene expression was analyzed in a wide variety of cells [54,55], the transcriptional control of *VEGF-A* gene in RPE cells has been unclear. Thus, we analyzed *VEGF-A* promoter and revealed that three SP1 binding sites in  $102$  to  $-64$  of the *VEGF-A* promoter was essential for *VEGF-A* transcription in RPE cells via SP1 binding to the 3 GC boxes.

SP1 is known as a transcription factor that binds to GC box sequence for transcriptional activation [56]. Previous studies supported the idea that SP1 plays a role not only in housekeeping type gene expression but also in inducible expression of various genes, such as phorbol ester-induced superoxide dismutase in endothelial cells [57], glucose-activated acetyl-CoA carboxylase promoter in preadipocytes [58], glucose-activated plasminogen activator inhibitor-1 promoter in vascular smooth muscle cells [59], and tumor necrosis factor  $\alpha$ -induced *VEGF* promoter in glioma cells [60]. In this study, we revealed that SP1 is the essential for the *VEGF-A* gene transcription in RPE cells. Deletion analysis of *VEGF-A* promoter demonstrated that the deletion from  $-102$  to  $-78$ ,  $-78$  to  $-64$  and  $-64$  to  $-43$  affected on the transcriptional activity of *VEGF-A* not only in control cells but also in HQ+AGE treated cells. On the other hand, RNA interference of SP1 significantly decreased *VEGF-A* gene expression in the HQ+AGE treated cells but not in untreated cells (Fig. 6). These results may suggest that the molecular mechanism for up-regulation of *VEGF-A* gene is different between the control cells and the HQ+AGE treated cells.

Based on the present study, we would like to propose a possible model of pathogenesis of dry- and wet-type AMD: Increases of HQ, which are frequently caused by cigarette smoking, induces apoptosis of RPE cells to lead patients dry-type AMD. In addition to HQ (cigarette smoking), increases of AGE, which are frequently caused by aging and/or diabetes, induce *VEGF-A* expression in RPE cells. *VEGF-A* acts not only as an autocrine growth factor to proliferate RPE cells but also as a paracrine growth factor to proliferate nearby vascular cells, resulting pathological neovascularization to lead patients wet-type AMD.

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## Appendix A. Supplementary information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.bbrep.2015.05.005>.

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