

Interactive Expressions of HtrA1 and VEGF in Human Vitreous Humors and Fetal RPE Cells

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PURPOSE. High-temperature requirement factor A1 (HtrA1) is associated with exudative age-related macular degeneration, an angiogenic retinal disease related to vascular endothelial growth factor (VEGF). This study investigates the interactive relationship between the expressions of HtrA1 and VEGF.

METHODS. The vitreous humor levels of HtrA1, VEGF, and pigment epithelium-derived factor were determined in 55 unrelated Han Chinese patients who underwent ocular surgeries. Expressions of *HTRA1* and *VEGFA* were studied interactively and under stress conditions in primary human fetal retinal pigment epithelial (RPE) cells to evaluate their regulations.

RESULTS. Vitreous levels of HtrA1 were significantly associated with that of VEGF in vitreous samples from all patients (Pearson's correlation coefficient test, $r = 0.650$, $P = 7.91 \times 10^{-8}$) and from patients with retinal detachment ($r = 0.835$, $P = 2.14 \times 10^{-7}$). On stress induction, *HTRA1* and *VEGFA* were upregulated in human fetal RPE cells treated by tunicamycin and dithiothreitol, but reduced after treatment by MG132. However, HtrA1 and VEGF did not regulate each other in their expressions.

CONCLUSIONS. This study revealed an association between HtrA1 and VEGF in human vitreous humors and RPE cells. They are both related to stress and inflammatory conditions. (*Invest Ophthalmol Vis Sci.* 2011;52:3706–3712) DOI:10.1167/iovs.10.6773

Angiogenic retinal diseases, such as age-related macular degeneration (AMD) and diabetic retinopathy, are major causes of irreversible visual impairment and blindness worldwide.¹ Among the known angiogenic factors, vascular endothelial growth factor (VEGF) is widely accepted as one of the most important regulators in physiological and pathologic angiogenesis.² VEGF, a dimeric secreted glycoprotein, is an endothelial cell-specific mitogen, a vascular permeability factor,³ and a cell survival factor.⁴ Results from studies on animal

models^{5,6} and anti-VEGF treatments for human patients with ocular vascular diseases^{7,8} have suggested that VEGF is one of the initiators or inducers of neovascularization in the eye. VEGF expression is elevated in the aqueous and vitreous humors of patients with different angiogenic ocular diseases.^{9,10} In addition, angiogenesis is a consequence of the equilibrium between stimulation by VEGF and inhibition by pigment epithelium-derived factor (PEDF).^{11,12} In the eye, PEDF downregulates VEGF expression and VEGF-induced vascular changes.^{13,14}

AMD, a retinal angiogenic disease associated with VEGF, affects approximately 50 million elderly people worldwide.¹⁵ We previously identified an association of exudative AMD with a single nucleotide polymorphism, rs11200638, in the promoter region of the high-temperature requirement factor A1 (*HTRA1*) gene.^{16–18} HtrA1 belongs to the evolutionarily conserved HtrA family of chymotrypsin-like serine protease, which exhibits temperature-dependent proteolytic and molecular chaperone activities.^{19,20} It carries a N-terminal secretory signal peptide, a mac25-like domain, and a C-terminal HtrA (proteolytic and PDZ) domain.^{21,22} Human *HTRA1* was identified as a differentially expressed gene in SV40-transformed fibroblasts²¹ and osteoarthritic cartilage.²² Downregulation of *HTRA1* was detected in 11–63% cancer samples from different tissues.^{23,24} In HtrA1-overexpressed cancer cell lines there was enhanced cell apoptosis and reduced cell proliferation.^{23,24} In contrast to cancer, upregulation of HtrA1 occurs in placenta-tion,²⁵ arthritis,^{22,26} Alzheimer's disease,²⁷ and Duchenne muscular dystrophy.²⁸ *HTRA1* mRNA and protein expressions were elevated in the lymphocytes and retinal pigment epithelium (RPE) of AMD patients carrying the risk-associated allele.¹⁷ HtrA1 is also present in drusen, abnormal RPE, and choroidal neovascularization lesion, with elevated expressions in AMD eyes.^{17,29–31} HtrA1 also inhibits TGF β signaling and degrades extracellular matrix proteins.^{26,32,33} These findings provide evidence for a contributory role of HtrA1 on AMD pathogenesis.

HtrA1 and VEGF are independently contributed to AMD. In this study we determined HtrA1, VEGF, and PEDF levels in human vitreous samples and examined *HTRA1* and *VEGF* expressions in cultured human fetal RPE cells under stress conditions, with a view to throwing light on their regulatory relationships.

MATERIALS AND METHODS

Study Subjects

Fifty-five unrelated Chinese patients who underwent ocular surgeries at the Prince of Wales Hospital in Hong Kong and the Joint Shantou International Eye Center (JSIEC) of Shantou University and the Chinese University of Hong Kong were recruited and given complete ophthalmoscopic examinations. In all patients, a standard three-port pars plana vitrectomy was performed as a part of the regular surgical procedures.

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TABLE 1. A Summary of Clinical Diagnosis, Gender, and Age of All 55 Unrelated Han Chinese Patients

Patients	Number (Percent of Total)
Total	55
Sex	
Male	33 (60.0%)
Female	22 (40.0%)
Clinical diagnosis*	
Ocular vascular diseases	12 (21.8%)
Retinal detachment	25 (45.5%)
Macular hole	8 (14.5%)
Trauma	7 (12.7%)
IOL-RO	2 (3.6%)
ERM	1 (1.8%)
Age in years; mean \pm SD	50.8 \pm 17

* Ocular vascular diseases included vitreous hemorrhage, diabetic retinopathy, and central retinal vein occlusion. Retinal detachment includes rhegmatogenous retinal detachment and recurrent retinal detachment. ERM, epiretinal membrane; macular hole, idiopathic macular hole; IOL-RO, intraocular lens reoperation; SD, standard deviation.

The clinical and demographic information is summarized in Table 1. None of the subjects had received treatment with anti-VEGF agents. The study protocol was approved by the Ethics Committee for Human Research at the Chinese University of Hong Kong and JSIEC and was in accordance with the tenets of the Declaration of Helsinki. Informed consent was obtained from the study subjects after explanation of the nature and possible consequences of the study.

Undiluted vitreous humor samples (0.5–1 mL) were collected into sterile tubes at the time of surgery, and aliquots were rapidly frozen at -80°C until assay. Peripheral venous blood samples (3 mL) were also collected and stored at -80°C before DNA extraction.

Immunoblotting

Total protein concentrations in the vitreous samples were measured by protein assay (BioRad, Hercules, CA). Equal amount of total protein (10 μg) for each denatured vitreous humor sample was resolved on 12.5% SDS-polyacrylamide gel and electro-transferred to nitrocellulose membranes for sequential probing with the mouse monoclonal antibodies against VEGF (Santa Cruz Biotechnology, Santa Cruz, CA), Htra1 (R&D Systems, Minneapolis, MN), and PEDF (Millipore, Billerica, MA) in the same blot and secondary antibody against mouse IgG conjugated with horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA). The signals were detected by enhanced chemiluminescence (Amersham Pharmacia, Cleveland, OH), and the band intensities were quantified (Quantity One Image Analysis software, BioRad). Triplicates were performed. The intensities of Htra1 and VEGF in the same blot were measured and compared directly. Therefore, normalization of a housekeeping protein was not needed.

HTRA1 rs11200638 Genotyping

The HTRA1 rs11200638 genotypes were determined in all 55 patients by PCR and direct DNA sequencing as previously described.¹⁸

Cloning of Human HTRA1

A 1440-bp open reading frame of the HTRA1 gene (NM 002775.4; GenBank) was cloned into an empty pcDNA6/myc-His A vector (Invitrogen, Carlsbad, CA) between the BamHI and NotI sites (pHis/myc-Htra1). The construct was verified by direct sequencing. Expression and proteolytic function of the recombinant human Htra1 protein were validated by immunoblotting and casein digestion, respectively.

Cell Culture Experiments

Primary human fetal RPE cells³⁴ were cultured in Dulbecco's modified Eagle's medium and F-12 nutrient mixture supplemented with $1\times$ penicillin streptomycin (Gibco BRL, Rockville, MD) and 10% heat-inactivated fetal bovine serum (Gibco BRL) at 37°C in a humidified environment containing 5% CO_2 . Passage 5–10 of human fetal RPE cells was used. Triple experiments were performed. To study cellular stress, confluent human fetal RPE cells were treated with tunicamycin (0.5, 5, and 10 $\mu\text{g}/\text{mL}$; Sigma-Aldrich, St. Louis, MO), dithiothreitol (DTT, 0.1, 1, and 2 mM; Sigma-Aldrich), and Z-Leu-Leu-Leu-H (MG132, 10 μM ; Sigma-Aldrich) in serum-free medium for 18 hours. For the VEGF regulatory experiments, confluent human fetal RPE cells were treated with recombinant human VEGF (10 ng/mL; Gibco BRL) in serum-free medium for 24 hours. For the Htra1 regulatory experiments, 70% confluent human fetal RPE cells were transfected with 3 μg pHis/myc-Htra1 construct in transfection reagent (Lipofectamine-2000, 9 μL ; Invitrogen) and incubated for 24 hours after transfection. Total RNA was collected at each time point with an extraction kit (RNeasy; Qiagen, Hilden, Germany).

Gene Expression Analysis

The RNA was reverse-transcribed to complementary DNA by reverse transcriptase (SuperScript III; Invitrogen) according to the manufacturer's instructions. The expressions of superoxide dismutase (SOD; stress maker),³⁵ interleukin-6 (IL6; inflammatory marker),³⁵ and HTRA1, VEGFA, and PEDF mRNA were analyzed using semiquantitative PCR (Table 2). β -ACTIN was used as a housekeeping gene for normalization. The PCR products were resolved in agarose gel and quantified (Quantity One Image Analysis software; BioRad) for comparison of relative band intensities.

Statistical Analysis

The χ^2 test was used to calculate the statistical significance among categorical parameters, the Pearson's correlation coefficient to measure linear associations, and the Spearman's rank correlation test to measure the association between rank orders. With reference to reported methods,^{23,24,36} the band intensities were categorized into

TABLE 2. Primers Used for Gene Expression Analysis

Gene Abbreviation	Primer Sequence	T_m ($^{\circ}\text{C}$)	Cycles
HTRA1	F: CAAAGCCAAAGAGCTGAAGG R: ACCATGTTTCAGGGTGCCTTC	60	28
VEGFA	F: GAGCCTTGCCTTGCTGCTCTA R: CACCAGGGTCTCGATTGGAT	60	28
PEDF	F: CAGTGTGCAGGCTTAGAGGGACTA R: AGGGTCTTGGCAGCTGCTGT	60	31
β -ACTIN	F: CAACGGCTCCGGATGTGC R: CTCTTGTCTGGGCTCGT	60	21

Cycles, number of PCR cycles; F, forward primer; R, reverse primer; T_m , annealing temperature.

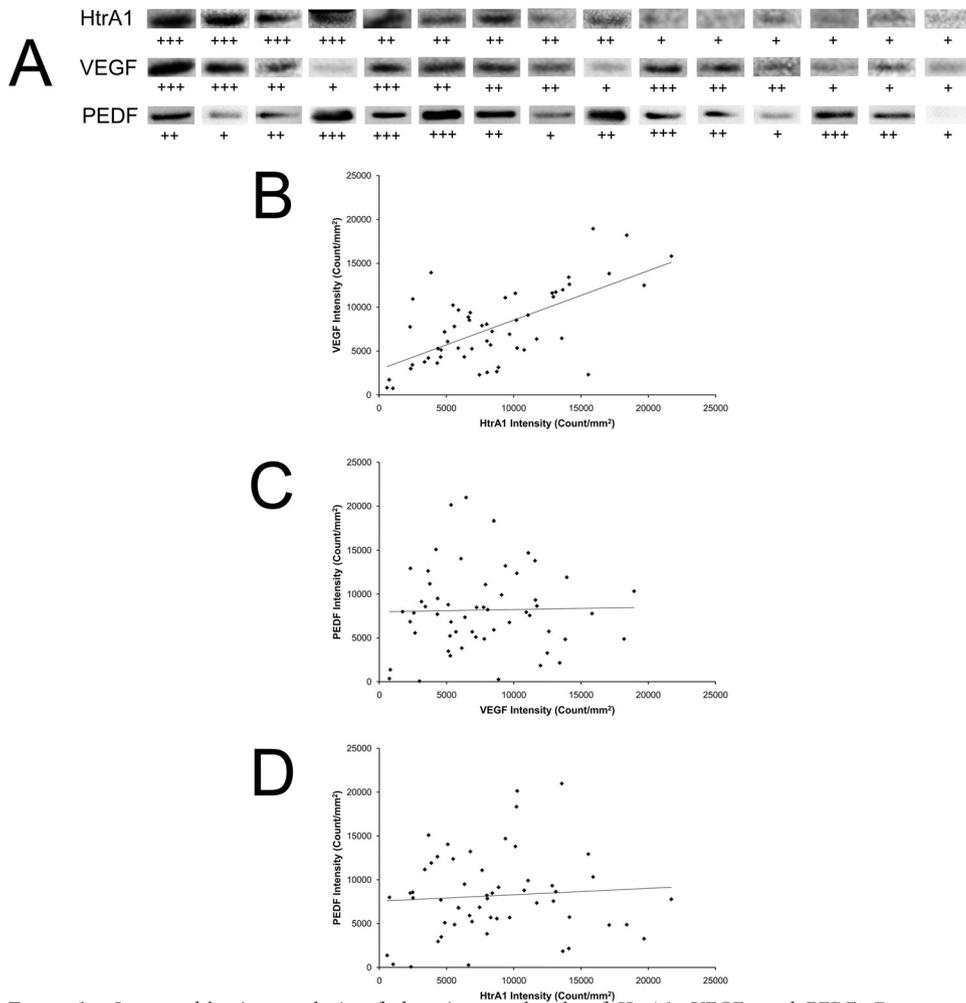


FIGURE 1. Immunoblotting analysis of the vitreous levels of HtrA1, VEGF, and PEDF. Denatured vitreous humor samples, all with 10 μ g total protein, were analyzed by immunoblotting with sequential probing of monoclonal antibodies against VEGF, HtrA1, and PEDF in the same blot. The signals were detected by enhanced chemiluminescence and quantified (Quantity One Image Analysis software; BioRad). (A) HtrA1, VEGF, and PEDF expressions were detected in vitreous humor at different intensities (+, weak; ++, moderate; +++, strong). (B) Significant association existed between vitreous levels of HtrA1 and VEGF (Pearson's correlation coefficient test, $r = 0.650$, $P = 7.91 \times 10^{-8}$). (C) PEDF was not associated with VEGF ($r = 0.023$, $P = 0.865$). (D) PEDF was also not associated with HtrA1 ($r = 0.077$, $P = 0.575$).

three groups for association analysis: weak (+) expression was defined as the vitreous humor sample distributed lower than the 25 quartile, moderate (++) as the 25-75 quartile, and strong (+++) as higher than the 75 quartile. All analyses were performed using commercially available software 0 (SPSS v. 16; SPSS, Chicago, IL). Significance was defined as $P < 0.05$.

RESULTS

Association of HtrA1 with VEGF in Human Vitreous Humors

HtrA1, VEGF, and PEDF were constitutively expressed in all vitreous humor samples as detected by immunoblotting (Fig. 1A). HtrA1 levels were significantly associated with VEGF (Pearson's correlation coefficient test, $r = 0.650$, $P = 7.91 \times 10^{-8}$; Fig. 1B). However, there was no association for PEDF with VEGF or HtrA1 (Pearson's correlation coefficient test, $r = 0.023$, $P = 0.865$ and $r = 0.077$, $P = 0.575$, respectively; Figs. 1C and 1D). After categorization of the HtrA1, VEGF, and PEDF expressions into three groups (weak, moderate, and strong)

according to a semiquantitative visual assessment of the expression levels, the association between the grouped vitreous humor levels of HtrA1 and VEGF remained significant (χ^2 test, $P = 9.09 \times 10^{-10}$; Spearman's rank correlation test, $\rho = 0.668$, $P = 2.55 \times 10^{-8}$; Table 3). However, the vitreous level of

TABLE 3. Correlation of Vitreous HtrA1 Levels with Vitreous VEGF Levels in All Patients

Grouped HtrA1 Level*	Grouped VEGF Level* n (Percent of Total Patients)		
	+	++	+++
+	9 (16.4%)	3 (5.5%)	1 (1.8%)
++	4 (7.3%)	23 (41.8%)	1 (1.8%)
+++	1 (1.8%)	2 (3.6%)	11 (20.0%)

* +, weak expression; ++, moderate expression; +++, strong expression.

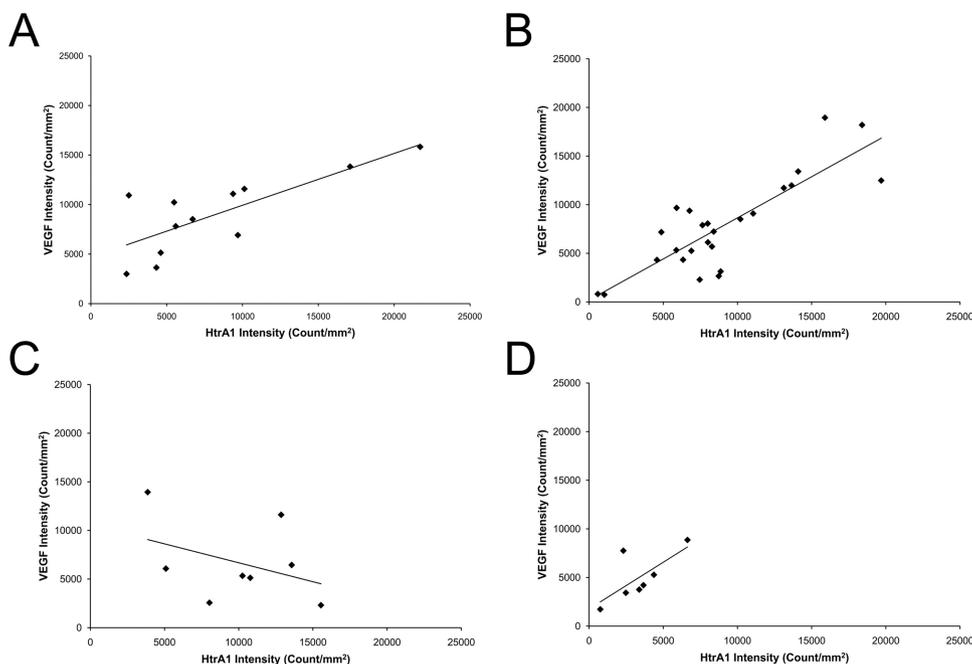


FIGURE 2. Correlation of the vitreous HtrA1 and VEGF levels in patients of different clinical diagnosis. The clinical diagnosis was categorized into five groups: vascular diseases, retinal detachment, idiopathic macular hole, and traumatic injury. The samples from patients with unclear diagnosis, ERM and IOL-RO, were excluded. Vitreous HtrA1 levels were associated with vitreous VEGF levels (A) significantly in retinal detachment (Pearson's correlation coefficient test, $r = 0.835$, $P = 2.14 \times 10^{-7}$) and (B) mildly in vascular diseases ($r = 0.778$, $P = 0.003$). No association was observed in (C) macular hole ($r = -0.390$, $P = 0.340$) and (D) traumatic injuries ($r = 0.706$, $P = 0.077$).

PEDF was still not associated with that of VEGF or HtrA1 (χ^2 test, $P = 0.765$ and 0.607 , respectively; Spearman's rank correlation test, $\rho = -0.145$, $P = 0.292$, and $\rho = -0.110$, $P = 0.422$, respectively). In addition, the vitreous levels of HtrA1, VEGF, and PEDF were not related to gender or age (data not shown).

The 55 patients, from whom we collected vitreous, were categorized into five groups according to the major disease phenotype: ocular vascular diseases, retinal detachment, idiopathic macular hole, and traumatic injury. The samples from patients with unclear diagnosis, that is, epi-retinal membrane (ERM) and intraocular lens reoperation (IOL-RO), were excluded from data analysis (Table 1). Vitreous HtrA1 was positively associated with vitreous VEGF in patients with retinal detachment (Pearson's correlation coefficient test, $r = 0.835$, $P = 2.14 \times 10^{-7}$) and mildly in patients with ocular vascular diseases (Pearson's correlation coefficient test, $r = 0.778$, $P = 0.003$; Figs. 2A and 2B). No association was observed in patients with macular hole or traumatic injuries (Pearson's correlation coefficient test, $r = -0.390$, $P = 0.340$ and $r = 0.706$, $P = 0.077$, respectively; Figs. 2C and 2D). In addition, the *HTRA1* rs11200638 genotypes were not associated with vitreous HtrA1 levels in all patients (χ^2 test; $P = 0.529$; Spearman's rank correlation test, $\rho = 0.129$, $P = 0.368$; Table 4) or in patients of each disease group, even after age-adjustment (data not shown).

Association of HtrA1 with VEGF in Human Fetal RPE Cells

Association of endogenous *HTRA1* and *VEGFA* was detected in human fetal RPE cells in culture under stress conditions. All the chemical-treated cells showed stress response, as indicated by the upregulation of *SOD* (Fig. 3). Moreover, inflammatory response (*IL6* upregulation) was also induced in cells treated with tunicamycin and DTT (Fig. 3). *HTRA1* and *VEGFA* expres-

sions were simultaneously upregulated in human fetal RPE cells treated by tunicamycin or DTT in a dose-dependent manner (Fig. 4). Downregulation of *HTRA1* and *VEGFA* expressions was found after MG132 treatment, suggesting that MG132 might override cell stress and regulate their gene expressions. Furthermore, no differential expression of *HTRA1* and *VEGFA* was detected in the overexpression experiments or exogenous treatments (Fig. 5). HtrA1-transfected cells showed sevenfold elevation in *HTRA1* expression but similar *VEGFA* and *PEDF* expressions when compared to the empty vector control (Figs. 5A and 5B). *HTRA1* and *VEGFA* expressions in VEGF-treated cells were not different from that in control (Figs. 5C and 5D). The results suggested that HtrA1 and VEGF did not directly regulate each other.

DISCUSSION

We speculated that expression of *HTRA1*, an AMD-associated gene,¹⁶⁻¹⁸ could be associated with that of VEGF, which is the

TABLE 4. Correlation of Vitreous HtrA1 Levels with *HTRA1* rs11200638 Genotypes

Grouped HtrA1 Level*	rs11200638 Genotype n (Percent of Total Patients)		
	GG	GA	AA
+	4 (7.8%)	8 (15.7%)	1 (2.0%)
++	4 (7.8%)	14 (27.5%)	8 (15.8%)
+++	3 (5.9%)	6 (11.8%)	3 (5.9%)

* +, weak expression; ++, moderate expression; +++, strong expression.

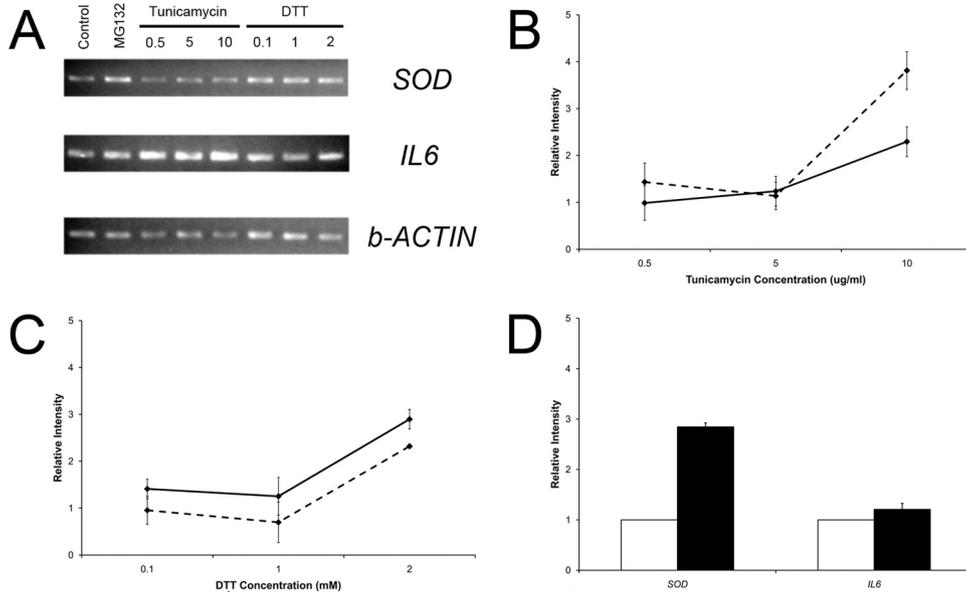


FIGURE 3. *SOD* and *IL6* expression analysis in stress-induced human fetal RPE cells. Confluent human fetal RPE cells were treated with tunicamycin (0.5, 5, and 10 $\mu\text{g}/\text{mL}$), DTT (0.1, 1, and 2 mM), or MG132 (10 μM) for 18 hours. Total RNA was collected and reverse-transcribed. (A) The gene expression levels of *SOD*, *IL6*, and β -*ACTIN* were analyzed by semiquantitative PCR. (B) Both *SOD* (solid line) and *IL6* (dotted line) were upregulated in 10 $\mu\text{g}/\text{mL}$ tunicamycin. (C) Both *SOD* and *IL6* were upregulated in 2 mM DTT. (D) Only *SOD* was regulated in MG132. White bar: gene expressions in control; black bar: gene expressions after MG132 treatment.

main factor of ocular angiogenesis. We have detected, for the first time, an association of HtrA1 and VEGF levels in human vitreous humor (Fig. 1), suggesting that they have correlated biological functions, especially in ocular diseases.

The association of HtrA1 and VEGF vitreous levels in retinal detachment (Fig. 2) might be related to inflammatory and stress responses.³⁷⁻³⁹ HtrA1 is associated with geographic atrophy²⁹ and drusen,^{30,31} and is upregulated in response to estrogen-induced oxidative stress.⁴⁰ We also found that human fetal RPE cells responded to chemical-induced stress by simultaneous up-regulations of *HTRA1* and *VEGFA* after treatments with tunicamycin and DTT (Figs. 4B and 4C). Although HtrA1 and VEGF might not be directly regulated by each other (Fig. 5), cellular stress could be a common and indirect regulatory pathway for HtrA1 and VEGF, substantiating the proposition that cellular stress could be a pathologic condition in AMD.^{41,42}

Notably, in the presence of another stress inducer, MG132,^{43,44} *HTRA1* and *VEGFA* were both downregulated (Fig. 4D). This suggested that MG132 might override the effect of cellular stress to regulate HtrA1 and VEGF. Proteasome is the direct inhibitory target of MG132,⁴⁵ which in turn inhibits a wide range of signaling pathways, including NF κ B. We therefore propose that HtrA1, together with VEGF, could be regulated by the NF κ B-dependent pathway, because human papillomavirus-type 16 E7 protein enhances NF κ B activity⁴⁶ and transcriptionally upregulates *HTRA1*.⁴⁷ Moreover, TGF β 2-induced VEGF expression is completely abrogated by the inhibitor of NF κ B.⁴⁸ In addition, NF κ B induces the expression of genes involved in chronic and acute inflammatory responses,⁴⁹ which concurred with the association of both HtrA1 and VEGF with inflammation.^{38,50} Further studies should be conducted to validate this hypothesis.

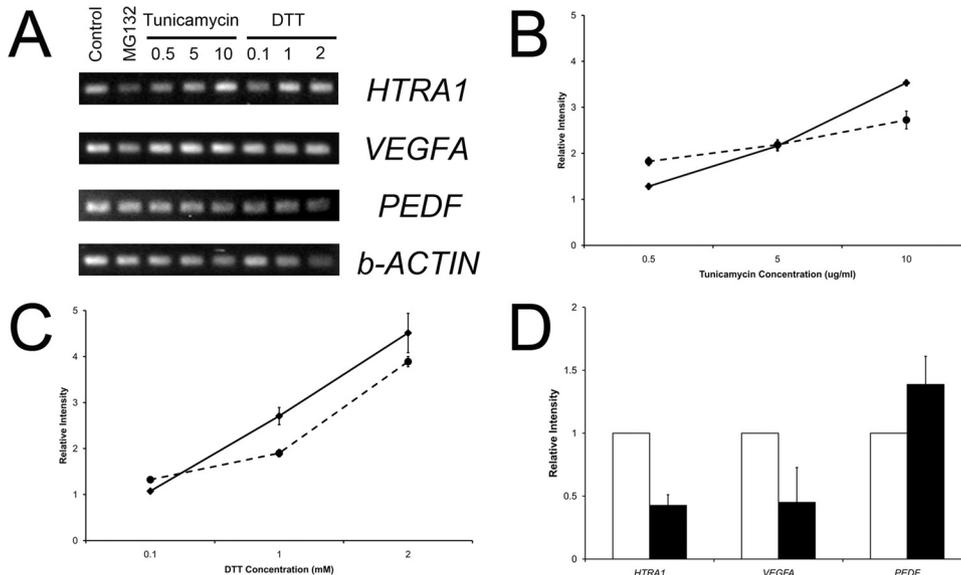


FIGURE 4. *HTRA1* and *VEGFA* expression analysis in stress-induced human fetal RPE cells. (A) Gene expression levels of *HTRA1*, *VEGFA*, *PEDF*, and β -*ACTIN* were analyzed by semiquantitative PCR. The *HTRA1* (solid line) and *VEGFA* (dotted line) expressions showed a dose-dependent increase in (B) 0.5–10 $\mu\text{g}/\text{mL}$ tunicamycin and (C) 0.1–2 mM DTT treatments. (D) *HTRA1* and *VEGFA* expressions were reduced in the cells treated with 10 μM MG132. White bar: gene expressions in control; black bar: gene expressions after MG132 treatment.

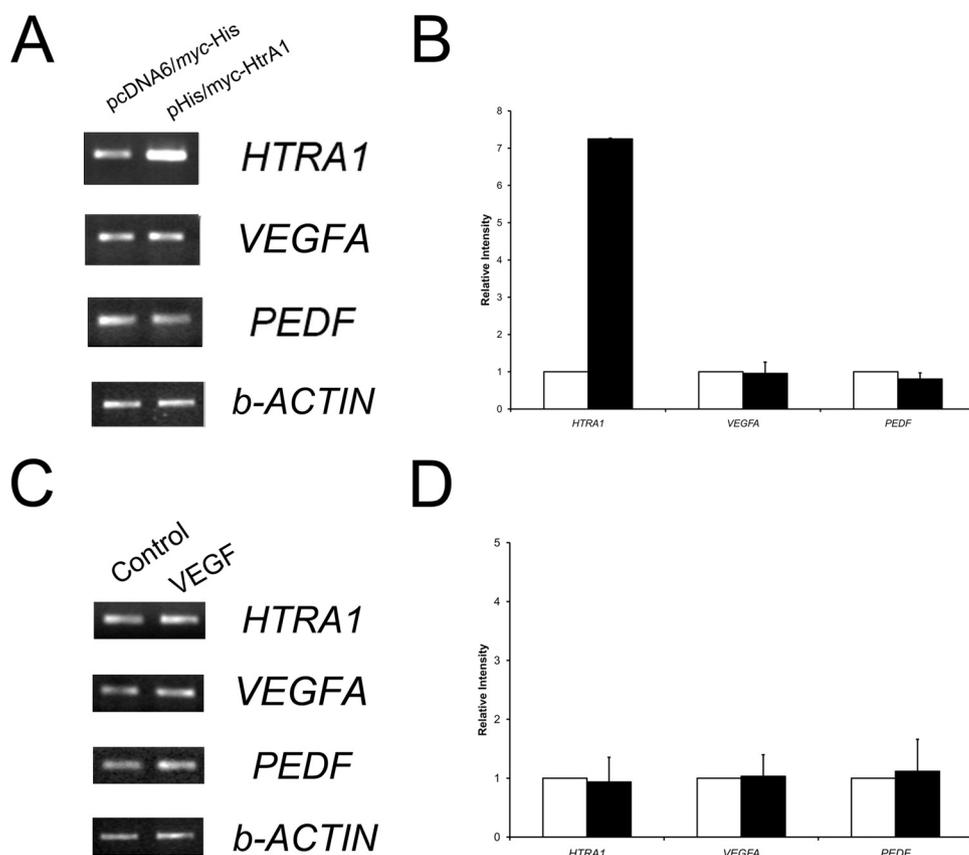


FIGURE 5. Gene expression study of Htra1-transfected and VEGF-treated human fetal RPE cells. Human fetal RPE cells were transfected with pHis/myc-Htra1 or treated with 10 ng/mL recombinant human VEGF for 24 hours. Total RNA was collected and reverse-transcribed. The expressions of *HTRA1*, *VEGFA*, *PEDF*, and β -*ACTIN* were analyzed by semiquantitative PCR. Htra1-transfected cells showed sevenfold elevated *HTRA1* expression, but similar *VEGFA* and *PEDF* expressions, compared to the empty vector control (A, B). The expressions of *HTRA1*, *VEGFA*, and *PEDF* in VEGF-treated cells were not different from that in untreated control (C, D). (B, D) White bar: gene expressions for empty vector control or untreated control; black bar: gene expressions for Htra1-transfected or VEGF-treated cells.

Unlike the previous studies,^{16,17} we found no association between vitreous Htra1 levels and rs11200638 genotypes (Table 4). It is likely that other factors regulating Htra1 expression might counteract with the effect of rs11200638 genotype on Htra1 expression. Indeed, *HTRA1* levels had been reported not having an association with rs11200638 genotypes.^{51,52}

Htra1 negatively regulates the availability of TGF β family through binding to its family members^{32,53} and inhibits TGF β -induced matrix synthesis in chondrocytes.⁵⁴ However, the direct role of Htra1 in angiogenic or inflammatory conditions is unclear. It is possible that Htra1 regulates the complement pathway and amyloid deposition by cleaving the RPE-secreted proteins.⁵⁵ In addition, the fragmented fibronectin product of the potential Htra1 extracellular matrix substrate would induce the release of cytokines.⁵⁶ Nevertheless, the results of this study showed a reduction of Htra1 and VEGF expressions induced by MG132, suggesting a possible therapeutic strategy against inflammatory diseases, such as AMD.⁴⁵

In summary, our results reveal an association between Htra1 and VEGF in human vitreous humors and RPE cells. They are both related to stress and inflammatory conditions.

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