HTRA1 and TGF-β1 Concentrations in the Aqueous Humor of Patients With Neovascular Age-Related Macular Degeneration

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Purpose. The purpose of this study was to evaluate the expression of high-temperature requirement A serine peptidase 1 (HTRA1), TGF-β1, bone morphogenetic protein 4 (BMP4), growth differentiation factor 6 (GDF6), and VEGFA proteins in the aqueous humor of patients with naïve choroidal neovascularization (nCNV) secondary to AMD.

Methods. We measured by ELISA the concentrations of HTRA1, TGF-β1, BMP4, GDF6, and VEGFA in the aqueous humor of 23 patients affected by nCNV who received three consecutive monthly intravitreal injections of 0.5 mg ranibizumab. Samples were collected at baseline (before the first injection), month 1 (before the second injection), and month 2 (before the third injection). Twenty-three age-matched cataract patients served as controls.

Results. Bone morphogenetic protein 4 and GDF6 were not detectable in any samples. Baseline HTRA1 was higher than controls ($P < 0.0001$) and higher than both the month 1 ($P < 0.0001$) and the month 2 ($P < 0.0001$) values. Baseline VEGFA was higher than controls ($P < 0.0001$), not different from month 1 value ($P = 0.0821$), but higher than month 2 value ($P < 0.0001$). Baseline TGF-β1 was higher than controls ($P = 0.0015$) and not different from month 1 ($P = 0.129$) and month 2 values ($P = 0.5529$). No correlation was found in naïve patients between concentrations of HTRA1 and TGF-β1, HTRA 1 and VEGFA, or TGF-β1 and VEGFA.

Conclusions. In nCNV patients, HTRA1 and TGF-β1 were significantly higher compared to controls. After treatment, TGF-β1 was persistently elevated, while HTRA1 returned to control levels, suggesting the involvement of TGF-β1 and HTRA1 in neovascular AMD and a VEGFA-independent role for TGF-β1.

Keywords: HTRA1, TGF-β, VEGFA, age-related macular degeneration, choroidal neovascularization

Age-related macular degeneration (AMD) is a major cause of visual loss in the elderly in developed countries. Neovascular AMD (nAMD), a subtype of advanced AMD, is responsible for almost 90% of severe visual loss due to AMD.[1] Age-related macular degeneration, including nAMD, is a multifactorial disease whose pathogenesis has been linked to numerous environmental and genetic factors.[1] Through genome-wide linkage and association, two major loci have been described as being associated with an increased risk of AMD: the complement factor H (*CFH*) gene at 1q31 and the age-related maculopathy susceptibility 2 (ARMS2)/high-temperature requirement factor A1 (*HTRA1*) locus at 10q26.[2–5] In particular, single nucleotide polymorphisms (SNPs) of *HTRA1* have been associated with increased risk of nAMD in various ethnic groups.[4–7] Some studies have related specific *HTRA1* alleles with neovascular lesion size[8] and with the response to intravitreal anti-VEGF therapy in AMD patients,[9] although this association was not confirmed by others.[10] Additionally, overexpression of HTRA1 in the retinal pigment epithelium of transgenic mice induces the exudative form of AMD.[11,12] Nevertheless, the intraocular concentration of HTRA1 protein in human patients affected by nAMD has, to our knowledge, never been tested, and it remains to be elucidated whether the SNPs of *HTRA1* identified are causally linked to aberrant gene expression or whether they are simply genetic markers.[13] In fact, the overexpression of HTRA1 protein and mRNA associated with the *HTRA1* risk allele *rs11200638*, initially documented in human retinas from ex vivo studies,[14–16] was subsequently negated in multiple ex vivo and in vitro studies.[17–20]

HTRA1 protein acts as a serine protease and is involved in protein quality control and cell fate.[21] The postulated pathways through which HTRA1 might modulate AMD development include the regulation of extracellular matrix (ECM) proteoglycan degradation and the modulation of TGF-β family member activity.[5,14,21–25] Indeed, recent evidence has focused on the role of TGF-β in favoring choroidal neovascularization (CNV), thus advocating TGF-β blocking agents.[26,27] However, despite the

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proposed role for HTRA1 in various neovascular diseases,[12,22,28–31] its mechanism of action on the TGF-β pathway is still subject to debate. Both positive and negative effects of HTRA1 on the processing or degradation of TGF-β1 and TGF-β proteins have been proposed, with contrary effects on the development of pathologic neovascularization.[21–23,28,29] Moreover, Friedrich et al.[23] recently showed that isoforms translated from synonymous risk variants of the *HTRA1* gene inefficiently bind to and proteolyze TGF-β1, thus, against the current tendency, suggesting a protective role for HTRA1 in nAMD. In the present study, we evaluated the protein concentration of HTRA1, TGF-β1, bone morphogenetic protein 4 (BMP4), growth differentiation factor 6 (GDF6), and VEGFA in the aqueous humor of patients affected by naïve nAMD at baseline and after intravitreal anti-VEGF injection in order to clarify the role of these molecules in the development of CNV and AMD.

Methods

Subjects

This prospective observational study comprised 23 patients affected by active CNV secondary to AMD. All eyes were examined and treated between June 2015 and February 2016 at the Ophthalmology Unit of the Department of Medicine, Surgery and Neuroscience, Siena University Hospital, Siena, Italy, after approval from the institutional review board. All subjects were treated in accordance with the Declaration of Helsinki. Patients were treated after being informed of the nature, purpose, implications, and risks of the treatment and after having signed a consent form.

The study enrolled patients who presented with CNV secondary to AMD. The diagnosis was confirmed by fluorescein angiography (FA), indocyanine green angiography, and spectral domain optical coherence tomography.

Patient demographics, study eye characteristics, and treatment details were recorded, including best corrected visual acuity (BCVA) measured using Early Treatment Diabetic Retinopathy Study charts at a distance of 4 m at baseline and during the course of follow-up; CNV angiographic subtype (classic vs. occult); lesion size, determined by the greatest linear dimension measured by FA examination before treatment;[8] central macular thickness (CMT) at the baseline and each follow-up visit; and number of injections required during follow-up.

All patients received three initial monthly injections followed by a flexible pro re nata regimen. All patients were followed for at least 8 months from their first injection.

None of the patients had received any previous treatment for nAMD, nor had they undergone any previous ophthalmic surgery, except cataract removal. Cataract surgery had to have been performed at least 9 months prior to inclusion. Controls were constituted by age-matched patients undergoing cataract surgery. The exclusion criteria for controls were any ocular disease except cataracts and any previous ophthalmic surgery. Diabetes mellitus, use of immunosuppressive drugs, and malignant tumors at any location were exclusion criteria for both patients and controls.

Aqueous Humor Sample Collection

All patients with nAMD received three consecutive monthly intravitreal injections of 0.5 mg ranibizumab. Aqueous samples were collected at baseline (day of the first injection), month 1 (day of the second injection), and month 2 (day of the third injection).

Anterior chamber taps were performed in the operating room prior to each intravitreal injection (patients) and before cataract surgery (controls) to obtain aqueous samples for measurement of the expression of HTRA1, VEGFA, TGF-β1, BMP4, and GDF6. A 30-gauge needle was inserted into the anterior chamber and 0.15 to 0.2 mL of aqueous humor was collected, centrifuged to remove cells and debris, aliquoted, and frozen at –80°C until analysis.

Assessment of the HTRA1, VEGFA, TGF-β1, BMP4, and GDF6 Levels in the Patients' Aqueous Humor

The HTRA1, VEGFA, TGF-β1, BMP4, and GDF6 concentrations in the patients' aqueous humor were measured by ELISA using ELISA kits for human VEGFA, TGF-β1, and BMP4 (Quantikine ELISA kit no. DVE00, no. DB100B, and no. DBP400, respectively; R&D Systems, Minneapolis, MN, USA) and ELISA kits for human HTRA1and GDF6 (no. MBS2504576 and no. MBS2515203, respectively; MyBioSource, San Diego, CA, USA). Each assay was performed according to the manufacturer's instructions. For each measurement of HTRA1, BMP4, and GDF6 concentration, 25 μL of samples (50 μL for VEGFA) were diluted to 100 μL using sample diluent buffer just prior to the assay. To perform this multiplex analysis a single sample was analyzed for each measurement due to sample volume limitation. In fact, extraction of the quantity of aqueous humor necessary to perform duplicate analyses for all of the five molecules tested here would have caused a complete collapse of the anterior chamber. To activate latent TGF-β1 to the immunoreactive form, 25 μL of aqueous humor was treated with 12.5 μL of 1N HCl and incubated for 10 minutes at room temperature. The acidified samples were neutralized to pH 7.3 using 10.31 μL of 1.2N NaOH/0.5M HEPES and diluted to 100 μL using sample diluent buffer.

Statistical Analysis

The data analysis was performed using statistical software (Prism 6; GraphPad, San Diego, CA, USA). The data are presented as box and whisker plots displaying median, lower, and upper quartiles and the minimum-maximum. The data were evaluated using ANOVA. Differences in the protein concentrations among the groups were estimated using Student's *t*-test and the nonparametric Mann-Whitney *U* test. Two-tailed probabilities of less than 0.05 were considered significant. The significance of correlations between protein concentrations in the aqueous humor of naïve AMD patients and control samples was tested using Pearson's correlation coefficient.

Results

The study compared the levels of HTRA1, VEGFA, TGF-β1, BMP4, and GDF6 in the aqueous humor of 23 patients with exudative AMD. Twenty-three age-matched cataract patients constituted the control group. The mean age was 78.2 ± 7.3 years in patients affected by nAMD and 73.6 ± 4.8 in controls. Age did not differ significantly between the two groups ($P = 0.449$). The male-to-female ratio was 1:2.875 and 1:3.28 in patients and controls, respectively.

Concentrations of HTRA1, VEGFA, and TGF-β1 were detected and measured by ELISA in the aqueous samples analyzed, while the BMP4 and GDF6 concentrations detected were similar to background values in all the samples analyzed (data not shown).

Baseline HTRA1 concentration was higher in AMD patients than in controls (baseline AMD median = 42.77 ng/mL [range of variation = 19.86–135.09], controls median = 25.59 ng/mL [11.53–53.80]; $P < 0.0001$) and higher than both the month 1

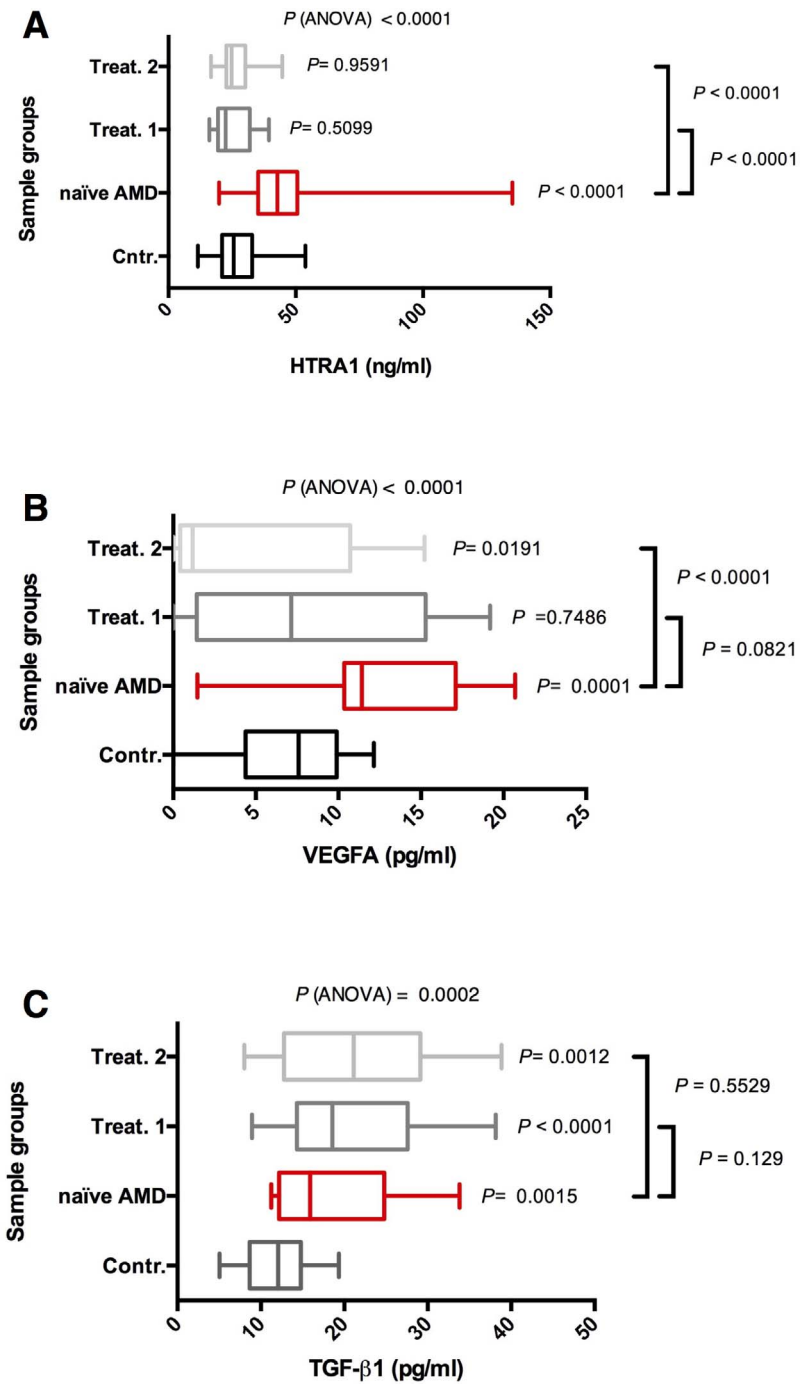

Figure 1. ELISA analysis of the aqueous levels of HTRA1, VEGFA, and TGF-β1. The (**A**) HTRA1, (**B**) VEGFA, and (**C**) TGF-β1 protein concentrations in the aqueous humor of patients and controls were determined by ELISA. Data are presented as box and whisker plots displaying median, lower, and upper quartiles (*boxes*) and minimum-maximum (*whiskers*). The *P* values beside each box were calculated by comparing naïve or treated (once or twice, Treat.1 and Treat.2, respectively) patient groups with a control group (Contr.). The *P* values calculated by comparing naïve with treated sample groups are indicated beside the *brackets*. The overall *P* value obtained by ANOVA is indicated above each graph.

(median = 22.44 ng/mL [16.04–36.82]; $P < 0.0001$) and the month 2 (median = 24.77 ng/mL [16,67–44.75]; $P < 0.0001$) values; HTRA1 month 1 and month 2 values were not different from controls (month 1, $P = 0.5099$; month 2, $P = 0.9591$). Baseline VEGFA was higher than in controls (baseline AMD median = 11.42 pg/mL [1.46–20.70], controls median = 7.59 [0–11.53]; $P < 0.0001$), not significantly different from month 1 value (median = 7.15 pg/mL [0–19.19]; $P = 0.0821$), but higher than month 2 value (median = 1.17 pg/mL [0–15.22]; $P < 0.0001$); VEGFA month 1 value was not significantly different from controls (month 1, $P = 0.7486$), while the month 2 value was lower than controls ($P = 0.0191$). Baseline TGF-β1 was higher than controls (baseline AMD median = 15.91 pg/mL [11.61–33.83], controls median = 12.07 pg/mL [5.03–19.34]; $P = 0.0015$) and not significantly different from month 1 (median = 18.56 pg/mL [8.94–38.15]; $P = 0.129$) or month 2 values (median = 21.11 pg/mL [8.02–38.85]; $P = 0.5529$); TGF-β1 month 1 and month 2 levels were higher than control values (month 1, $P < 0.0001$; month 2, $P = 0.0012$) (Fig. 1).

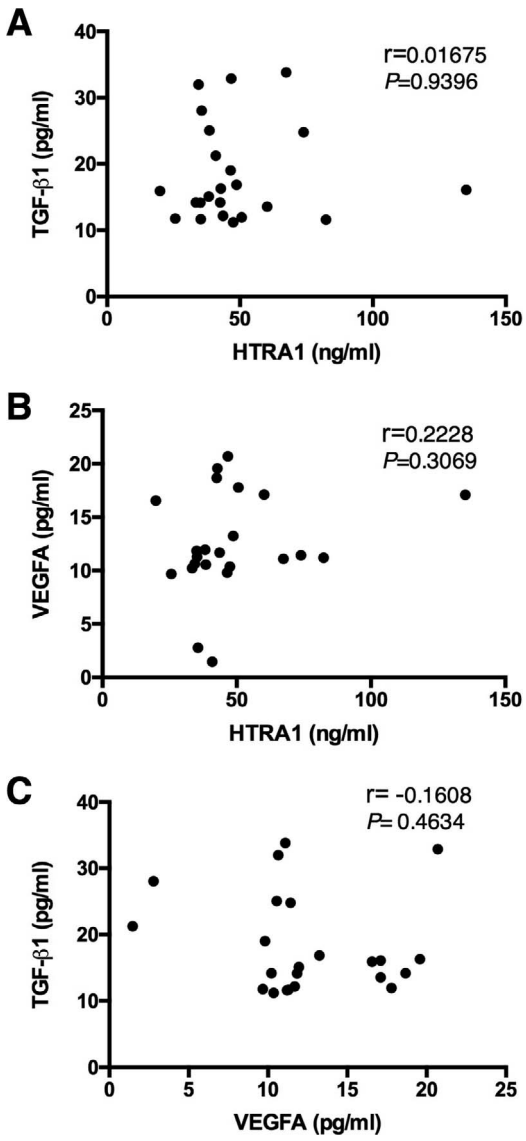

FIGURE 2. Correlation analysis of aqueous humor concentrations of HTRA1, VEGFA, and TGF-β1. Data are presented as scatter plots. In naïve AMD patients, no correlation was found between concentrations of (**A**) HTRA1 and TGF-β1, (**B**) HTRA1 and VEGFA, or (**C**) TGF-β1 and VEGFA. The correlation coefficient (*r*) was obtained using linear regression (Pearson's) analysis.

In naïve patients (at baseline) no correlation was found between the aqueous humor concentrations of HTRA1 and TGF-β1, HTRA1 and VEGFA, or TGF-β1 and VEGFA (Fig. 2).

Finally, considering the clinical characteristics of AMD patients analyzed herein, we did not find any significant correlation between HTRA1 concentration and any of the following: baseline BCVA ($P = 0.623$), BCVA change between baseline and final follow-up visit ($P = 0.391$), angiographic CNV type ($P = 0.095$), lesion size ($P = 0.675$), CMT at baseline ($P = 0.845$), change in CMT between baseline and last follow-up visit ($P = 0.535$), or the number of injections ($P = 0.583$).

DISCUSSION

This study analyzed the aqueous humor concentrations of HTRA1, TGF-β1, and VEGFA at baseline and after one and two intravitreal injections of ranibizumab in previously untreated patients affected by nAMD. At baseline, not only VEGFA but also HTRA1 and TGF-β1 concentrations were significantly higher compared to controls. Intravitreal injections of ranibizumab resulted in a significant reduction of VEGFA and HTRA1 concentrations, while TGF-β1 continued to be significantly raised.

Our data give evidence of the role of HTRA1 protein in nAMD, thus contributing potentially beneficial information regarding nAMD pathogenesis. In fact, although genome-wide association studies have linked *HTRA1* SNP to nAMD, the precise role of HTRA1 protein has remained elusive, since numerous studies have recently confuted the previously documented association between *HTRA1* risk alleles and the overexpression of HTRA1 protein and mRNA. Moreover, to our knowledge, the intraocular expression of HTRA1 protein in human patients affected by nAMD has never previously been tested.[14–20] HTRA1 acts as a serine protease, and as such, it might contribute to angiogenesis by multiple mechanisms, including the titration of growth factor activity and availability, the regulation of ECM composition and integrity, and the generation of angiogenically active ECM fragments.[24,32] Our data confirm the importance of proteases in ocular angiogenesis. In fact, the increased aqueous levels of HTRA1 detected are in line with the previously described increased vitreous concentration of metalloproteinases (MMPs) and aqueous concentration of cathepsin in patients with nAMD.[24] This might be of primary importance, since inhibitors of MMPs are negative regulators of naïve CNV.[24]

With regard to the HTRA1 and VEGFA levels, while we observed their similar trend in the sample groups, we did not find any correlation between them in the naïve untreated AMD samples, suggesting that HTRA1 and VEGFA did not directly regulate each other: This is consistent with the in vitro findings of Ng et al.[19]

One of the most documented functions of HTRA1 involves its association with the TGF-β family.[21–23,33,34] HTRA1 is thought to down-regulate TGF-β signaling both in AMD and in cerebral small vessel disease.[21–23,33] However, the inhibitory effect of HTRA1 on TGF-β has recently been confuted in cerebral small vessel disease.[34] TGF-β family members have a pleiotropic effect and may have different roles in the regulation of vascular endothelial and smooth muscle cells, being pro- or antiangiogenic in a context-dependent way.[35] However, recent and mounting evidence suggests a prominent role for TGF-β in favoring nAMD, thus advocating a TGF-β–blocking agent in nAMD treatment.[26] In the present series, we showed increased aqueous expression of TGF-β1 at baseline, confirming the data of Bai et al.[26] who documented increased vitreous TGF-β level in patients with nAMD. Interestingly, TGF-β1 expression was not correlated with the expression of either VEGFA or HTRA1 in naïve untreated AMD patients, and notwithstanding treatment, TGF-β1 levels remained significantly higher than in controls. This suggests that the levels of these proteins are not directly linked to one another and that each of them might contribute to nAMD genesis and development, but in an independent way, as previously suggested by the analysis of the mouse model overexpressing HTRA1.[30]

The increased concentration of TGF-β1 at baseline and the lack of a decrease in TGF-β1 following anti-VEGF intravitreal injections could lead us to hypothesize a potential beneficial effect on the disease course of nAMD of direct antagonism by TGF-β1. However, the present findings should be studied in greater depth through concomitant analysis of TGF-β2, which has been shown to be expressed in the aqueous humor under physiological conditions[36] and through quantification of the activated TGF-β among the total TGF-β protein concentrations.

The present study demonstrates a potential causative role of HTRA1 protein in nAMD and confirms the possibility of

blocking TGF-β1 as an additional treatment for nAMD. However, further studies using a larger number of samples are needed in order to verify this issue and to better understand the connection between HTRA1 and the TGF-β family members, as well as their precise role in nAMD.

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