Suppression of Choroidal Neovascularization by Thioredoxin-1 via Interaction with Complement Factor H

Yasuya Inomata,[1] *Hidenobu Tanihara,*[1] *Masaki Tanito,*[2] *Hiroaki Okuyama,*[3] *Yuma Hoshino,*[3] *Tomoya Kinumi,*[4] *Takahiro Kawaji,*[1] *Norihiko Kondo,*[5] *Junji Yodoi,*[5] *and Hajime Nakamura*[3]

Purpose. To examine the role of thioredoxin-1 (TRX-1), an endogenous protein with a variety of redox-related roles, in the formation of choroidal neovascularization (CNV).

Methods. CNV was induced by laser photocoagulation of the ocular fundus in wild-type and transgenic mice overexpressing human TRX-1 (TRX-1 Tg). Mice were injected intraperitoneally with TRX-1, mutant TRX, or vehicle. The incidence of CNV was evaluated by lectin staining. Leukocyte recruitment and C3b deposition after laser injury were determined by immunohistochemistry and Western blotting. Moreover, TRX-1–associated proteins from human plasma were isolated by two-dimensional gel electrophoresis with the use of a column coupled with a mutant TRX-1 and were identified by mass spectrometry and proteomics analysis. Complement activation was determined by a fluid-phase method.

Results. The incidence of laser-induced CNV was reduced in TRX-1 Tg mice (56.1%) and in C57B/6 mice treated with TRX-1 (46.7%) but not in mutant TRX-1 (79.2%) compared with wild-type mice (85.7%). Furthermore, leukocyte recruitment was prevented in TRX-1–treated mice; C3b deposition was decreased in these and TRX-1 Tg mice. In human plasma, five proteins associated with TRX-1 were identified as apolipoprotein A-I, the CD5 antigen-like member of the scavenger receptor, cysteine-rich superfamily fibrinogen, albumin, and complement factor H (CFH). TRX-1 inhibited the alternative pathway C3 convertase, and its effect was additive with CFH.

Conclusions. These findings show that TRX-1 interacts with CFH, regulates complement activity, and inhibits CNV, suggesting novel preventive and interventional therapeutic strategies for AMD. (*Invest Ophthalmol Vis Sci.* 2008;49:5118–5125) DOI:10.1167/iovs.07-1659

From the [1]Department of Ophthalmology and Visual Science, Kumamoto University Graduate School of Medical Sciences, Kumamoto, Japan; [2]Department of Ophthalmology, Shimane University School of Medicine, Shimane, Japan; [3]Thioredoxin Project, Department of Experimental Therapeutics, Translational Research Center, Kyoto University Hospital, Kyoto, Japan; [4]Human Stress Signal Research Center, National Institute of Advanced Industrial Science and Technology, Osaka, Japan; and [5]Department of Biological Responses, Institute for Virus Research, Kyoto University, Kyoto, Japan.

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Corresponding author: Hajime Nakamura, Thioredoxin Project, Department of Experimental Therapeutics, Translational Research Center, Kyoto University Hospital, 54 Shogoin-Kawaharacho, Sakyo, Kyoto, 606-8507; hnakamur@kuhp.kyoto-u.ac.jp.

Age-related macular degeneration (AMD) is the leading cause of legal blindness in industrialized countries.[1,2] Choroidal neovascularization (CNV), new vessel growth under the retina, is one of the major causes of serious visual loss in patients with AMD. The complex biological processes underlying the development of CNV and AMD are not completely understood. However, a growing body of evidence has suggested that the complement system might play a critical role in the development of CNV and that oxidative stress is widely involved in the progression of many age-related chronic degenerative diseases, including AMD.[3–5] Several studies have shown an association between variants in the factor H (*CFH*) and factor B (*CFB*) genes encoding major regulatory proteins in the alternative complement pathway and the risk for AMD.[6–9]

Thioredoxin (TRX-1) is a multifunctional protein with a redox-active dithiol/disulfide bond within its conserved active site (-Cys[32]-Gly-Pro-Cys[35]-). It catalyzes the reversible reduction of disulfide in organisms ranging from *Escherichia coli* to humans,[10,11] and its biological functions range from scavenging reactive oxygen species (ROS) in cooperation with peroxiredoxin to modulating its binding proteins, such as apoptosis signal-regulating kinase (ASK)-1[12] and TRX binding protein (TBP)-2.[13] We previously reported that the exogenous administration of recombinant human TRX-1 (rhTRX-1) attenuated acute inflammation,[14] acute lung injury,[15] and autoimmune myocarditis by mechanisms associated with the inhibition of leukocyte infiltration.[16]

Here we investigated the effect of TRX-1 on the formation of CNV in a commonly used mouse model of laser injury that triggers the proliferation and migration of choroidal endothelial cells by rupture of the Bruch membrane, as previously described.[4,5] Moreover, we report here that five proteins associated with TRX-1, including factor H (identified in human plasma and TRX-1), modulate complement activity on their own.

Methods

Animals

We purchased 12-week-old male C57BL/6 mice from Japan SLC (Tokyo, Japan). The TRX-1 Tg mice, in which human TRX-1 complementary DNA had been inserted between the β-actin promoter and the terminator sequences, have been described previously.[17] The presence of the TRX-1 transgene was confirmed by RT-PCR analysis before the experiments were conducted. All procedures were performed according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Induction of CNV

CNV was induced as previously described,[5] with minor modifications. Briefly, general anesthesia was induced by intraperitoneal injection (1 mL/kg) of a mixture of ketamine hydrochloride and xylazine hydrochloride. Pupils were dilated with one drop of 0.5% tropicamide (Santen Pharmaceutical, Osaka, Japan). Three or four burns were made with a YAG/green laser (532 nm, 50-μm spot size, 0.05-second dura-

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tion, 250 mW; GYC-1000; Nidek, Fremont, CA) on the retina close to the optic disc in mice. After 7 days, the mice were perfused with 20 mL PBS containing 50 μg/mL fluorescein-labeled tomato lectin (Vector Laboratories, Burlingame, CA) to stain the blood vessels by binding glycoproteins. Eyes were harvested and fixed in 4% paraformaldehyde, and flatmounts of the RPE-choroid-sclera were prepared and stained for elastin (Sigma, St. Louis, MO) in the Bruch membrane, followed by an Alexa-594–labeled secondary antibody (Molecular Probes, Eugene, OR), as previously described.[18] Tissues were washed and mounted in medium with the nuclear stain DAPI (Vector Laboratories). The incidence of CNV was determined by all-in-one-type fluorescence microscopy (BZ-8000; Keyence, Osaka, Japan).

TRX-1 Treatment

C57BL/6 mice were injected intraperitoneally with 10 or 100 μg rhTRX-1 (Redox Biosciences, Inc., Kyoto, Japan; Ajinomoto Co. Ltd., Kyoto, Japan), 6× histidine-tagged wild-type TRX-1 (WT-TRX-1), DM-TRX-1, or vehicle only simultaneously with laser photocoagulation and daily thereafter until the end of the experiment. WT-TRX-1 and DM-TRX-1 proteins were prepared as described previously.[19]

Leukocyte Recruitment

Three days after laser treatment, eyes were harvested and fixed in 4% paraformaldehyde, and RPE-choroid-sclera flatmounts were prepared. Neutrophils and monocytes/macrophages were stained using FITC-conjugated mAbs against LY-6G/LY-6C (BD PharMingen, San Diego, CA) and F4/80 (Serotec, Oxford, UK). FITC-conjugated rat IgG2b (Serotec) was used as a negative control. Tissue was treated with blocking solution (Dako Corp., Carpinteria, CA), washed, and stained with propidium iodide (Sigma). The margins of laser-induced lesions were determined as the breaking of RPE by light microscope and as the accumulation of propidium iodide staining. Leukocyte recruitment was measured as the volume of FITC labeling in the flatmounts using all-in-one type fluorescence microscopy (Keyence, Woodcliff Lake, NJ) with a 20× objective. Horizontal optical sections at 0.5-μm intervals were collected over 20 μm, from the surface of the RPE-choroid-sclera complex to the floor of the lesion, and stored digitally. The volume of FITC labeling was computed using the image analysis software supplied with the microscope (Keyence).

C3b Deposition

One day after laser treatment, eyes were enucleated, and flatmounts were prepared and stained with a mAb against C3b (Hycult Biotech, Uden, The Netherlands). After treating with blocking solution (Dako Corp.), FITC-conjugated anti–rat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) was used as the secondary antibody. Normal rat serum at concentrations similar to for the primary mAb was used as a control. In addition, controls that omitted the primary or secondary antibodies were routinely included. C3b deposition was measured as the volume of FITC and was assessed by Western blotting in eyes removed 1 day after laser treatment. RPE-choroid-sclera tissue was homogenized in radioimmunoprecipitation assay buffer. Homogenates were centrifuged at 20,000*g* for 15 minutes at 4°C, and protein concentrations in the supernatants were determined using a protein assay kit (DC; Bio-Rad Laboratories, Richmond, CA). Equal amounts of protein (10 μg/lane) were separated on 10% SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride membranes. Membranes were blocked in Tris-buffered saline containing 0.1% Tween 20 and 5% BSA and were incubated overnight at 4°C with 1:200 dilutions of primary antibodies against C3b and then with a peroxidase-linked secondary antibody (Amersham Pharmacia, Buckinghamshire, UK). Chemiluminescence was detected with a Western blot kit (ECL; Amersham Pharmacia).

Isolation and Proteomic Analysis of TRX-1–Associated Proteins in Human Plasma

C35S-TRX-1 was coupled to CNBr-activated Sepharose 4B (Amersham Pharmacia) according to the manufacturer's instructions. Human plasma, collected by centrifuging heparinized venous whole blood, was applied to the C35S-TRX-1–coupled column. As a control, human plasma preincubated with rhTRX-1 was applied to the same column. The column was extensively washed with 50 mM Tris-HCl, 1 mM EDTA buffer containing 0.5 M NaCl, and 1 mM EDTA buffer containing 1 M NaCl to remove noncovalently bound proteins. Subsequently, the potential target proteins were eluted with buffer containing 10 mM dithiothreitol (DTT). Eluted proteins were precipitated by the addition of 5 volumes of cold methanol and incubated at −20°C overnight. To separate the proteins by two-dimensional gel electrophoresis, the precipitates were dissolved in isoelectric focusing buffer (9 M urea, 65 mM DTT, 2% CHAPS, and 0.5% ampholyte; Amersham Pharmacia) and separated on immobilized pH gradient (IPG) strips (7-cm long, pH 3–10; Amersham Pharmacia). After isoelectric focusing, the IPG strips were equilibrated in SDS-equilibration buffer (6 M urea, 2% SDS, 20% glycerol, 50 mM Tris-HCl buffer, pH 6.8) and separated in the second dimension on 12.5% SDS polyacrylamide gels and stained (SyproRuby; Invitrogen, Carlsbad, CA).

Spots that decreased in intensity when rhTRX-1 was added to human plasma were excised, digested with trypsin, and identified by capillary liquid chromatography-tandem mass spectrometry, as described previously.[20] Mass spectra and tandem mass spectra were submitted to the MASCOT search software (Matrix Science, London, UK), and the National Center for Biotechnology Information Nonredundant Protein Database (NCBI nr) was used to identify the proteins.

Western Blot Analysis of TRX-1 and Factor H Interactions

We confirmed the interaction between TRX-1 and factor H by Western blotting. Mixtures of TRX-1 plus human plasma or TRX-1 plus purified factor H (Calbiochem, San Diego, CA), at varying concentrations, were incubated at 37°C for 60 minutes. Equal volumes were separated on 10% SDS-polyacrylamide gels and then were silver stained or transferred for Western blotting using anti–factor H (Abcam, Cambridge, UK) or anti–TRX-1 mAbs.

C3 Convertase Measurement

C3 convertase activity was measured as described previously, with some modifications.[21] Briefly, the C3 convertase was assembled by adding 10 μg C3 (Sigma), 50 ng C3i, and 2 μg factor B (Calbiochem) in the presence of Mg^{2+}. C3i, also known as C3(H_2O), was generated by five freeze/thawing cycles of purified C3. Varying concentrations of TRX-1, WT-TRX-1, DM-TRX-1, and factor H were added, followed by 200 ng factor D (Calbiochem), and the mixture was incubated at 37°C for 30 minutes. The generation of C3a was measured by ELISA (Quidel, San Diego, CA). All experiments included a positive control (C3, C3i, factor B, and factor D) and a negative control (C3, C3i, and factor B).

Statistical Analysis

Data are presented as mean ± SD for at least four independent experiments. Statistical comparisons of multiple groups used one-way analysis of variance (ANOVA) followed by Scheffé post hoc test. Comparisons of two groups used χ^2 test, Student's *t*-test, or Mann-Whitney *U* test. $P < 0.05$ was considered significant.

Results

Suppression of CNV by Administration or Overexpression of TRX-1

As shown in Figures 1A and 1B, 7 days after laser injury, neovascular complexes characteristic of CNV were observed in the subretinal spaces. In wild-type C57B/6 mice, the incidence of CNV was 85.7%, whereas in Tg mice overexpressing human

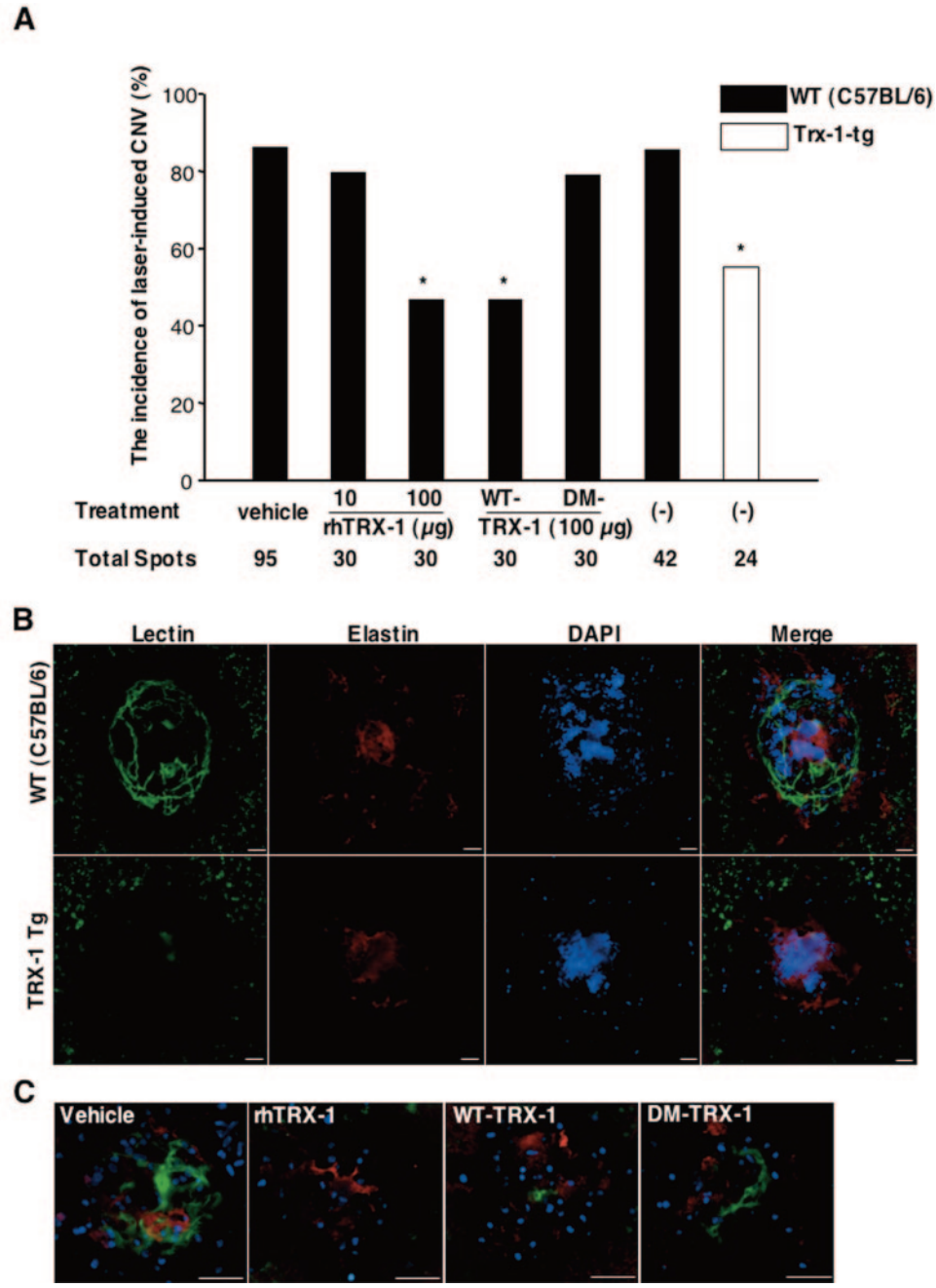


FIGURE 1. Effects of TRX-1 on laser-induced CNV in mice. Seven days after laser injury, the incidence of CNV was determined (**A**). The statistical significance of differences between groups was calculated by a χ^2 test. $^*P < 0.05$. Representative micrographs of CNV lesions 7 days after laser injury, stained with FITC-lectin for vessels, elastin, and DAPI in wild-type C57BL/6 mice (**B**, *upper*) and in TRX-1 Tg mice (**B**, *lower*) or in mice treated with vehicle, rhTRX-1, WT-TRX-1, and DM-TRX-1 (**C**). Scale bars, 50 μm.

TRX-1, under the control of a β-actin promoter, the incidence was significantly lower, 56.1% (Figs. 1A, 1B). The incidence of CNV was also significantly reduced in wild-type C57B/6 mice treated with 100 μg recombinant human TRX-1 (rhTRX-1; 46.7%) but not in mice treated with a double-mutant form of TRX-1 (DM-TRX-1; 79.2%) in which the cysteine in the active site had been replaced by serines (Figs. 1A, 1C). Thus, TRX-1 effectively inhibited the development of CNV, and a functionally active site was critical for its effect.

Inhibition of Leukocyte Recruitment and Complement Activation by TRX-1

To further investigate the anti-inflammatory effects of TRX-1, we examined the recruitment of leukocytes to sites of CNV, 3 days after laser injury, by fluorescence microscopy (Fig. 2A). Although the infiltration of neutrophils and monocytes/macrophages was clearly observed in vehicle-treated wild-type mice, their recruitment was markedly inhibited in the rhTRX-1–treated mice (Figs. 2B, 2C). Because complement activation plays a crucial role in the development of laser-induced choroidal neovascularization,[18] we also looked at the deposition of C3b, which was expected to occur as the result of complement activation. In flatmounts of the laser injury lesions, staining for C3b was clearly observed around the lesion in wild-type and vehicle-treated mice 1 day after laser injury, but C3b labeling was weak in TRX-1 Tg- and rhTRX-1–treated wild-type mice (Figs. 2D, 2E). Similar results were observed when C3b deposition was analyzed by Western blotting analysis (Fig. 2F). We concluded that TRX-1 inhibited the complement activation and leukocyte infiltration induced by laser injury and suppressed CNV development and that the active site was critical for its effects.

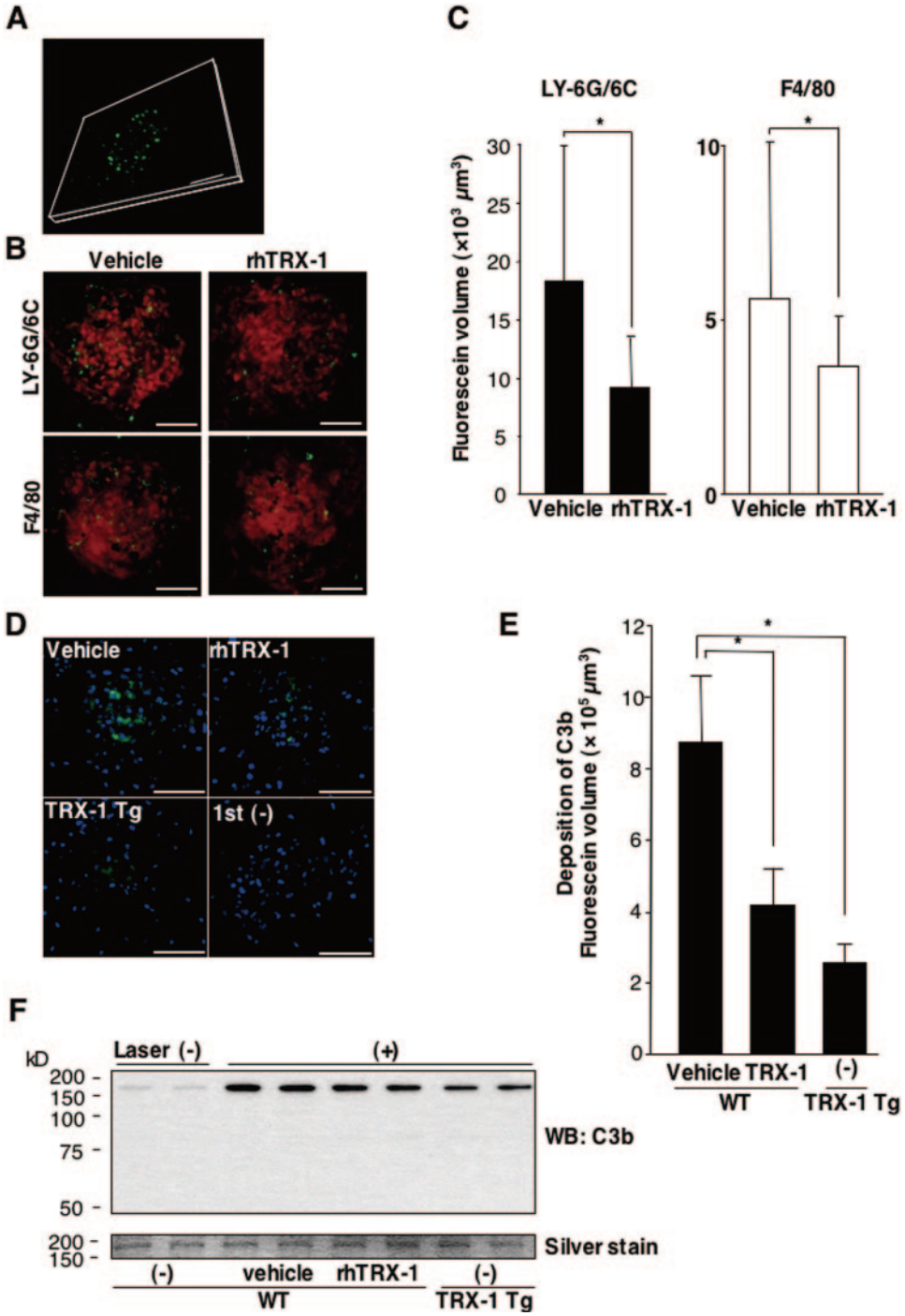
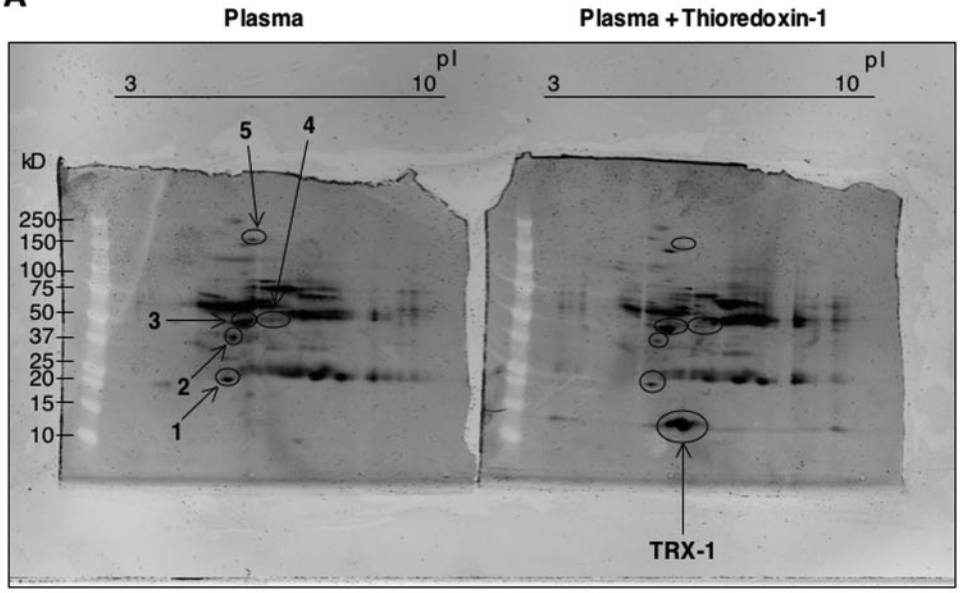


FIGURE 2. TRX-1 prevents leukocyte recruitment and C3b deposition within laser-induced lesions. A representative three-dimensional image of a lesion 3 days after laser injury (**A**) showing infiltrating neutrophils stained with FITC-anti-LY6G/6C mAb. Infiltrating neutrophils and macrophages in a flatmount of eyes treated with vehicle or rhTRX-1, stained with FITC–anti-LY6G/6C and FITC–anti-F4/80 mAbs (**B**). Reduced infiltration of neutrophils and macrophages in rhTRX-1–treated mice compared with vehicle-treated mice, evaluated as fluorescence volume within the laser-induced lesions, labeled with anti–LY6G/6C and anti–F4/80 mAbs (**C**). Data are mean ± SD ($n = 9$; $^*P < 0.05$). Representative flatmounts from C57B/6 mice treated with vehicle or rhTRX-1 or from TRX-1 Tg mice, stained with an anti–C3b mAb and an FITC-conjugated secondary antibody, 1 day after laser injury (**D**). Reduced deposition of C3b in rhTRX-1–treated C57B/6 mice or TRX-1 Tg mice compared with vehicle-treated mice, evaluated as fluorescence volume within the laser-induced lesions, labeled with anti-C3b mAb (**E**). Data are mean ± SD ($n = 9$; $^*P < 0.05$). Western blot analysis showed C3b in wild-type mice treated with vehicle or rhTRX-1 and in TRX-1 Tg mice 1 day after laser injury (**F**). Scale bars, (**A**, **B**, **D**) 50 μm.

Identification of Five Proteins That Interact with TRX-1 in Human Plasma

To investigate the molecular mechanisms underlying the anti-inflammatory effects shown by TRX-1 in the eye, we identified the molecules in plasma that interacted with it. A column coupled with a mutant TRX-1, in which the cysteine at position 35 was replaced with serine (C35S-TRX-1), was used to isolate TRX-1–associated proteins from human plasma. This method using C35S-mutant thioredoxin was previously reported to identify the target proteins for plant thioredoxin.[22] Analysis of the bound proteins by two-dimensional gel electrophoresis identified five spots, the intensities of which were reduced when the human plasma had been preincubated with rhTRX-1 (Fig. 3A). These spots were excised, digested with trypsin, and identified by mass spectrometry and proteomics analysis (Fig. 3B) as apolipoprotein A-I (the CD5 antigen-like member of the scavenger receptor cysteine-rich superfamily), fibrinogen, gamma polypeptide, and complement factor H. The last of these has crucial roles as a negative regulator of the alternative complement pathway, as a direct inhibitor of the cleavage of C3 to C3a and C3b, and as a competitor for factor B binding to C3b to form C3 convertase.[23] Given that recent work has indicated an association between variants of the *CFH* gene and AMD,[6–8] we focused our studies on the interaction between TRX-1 and factor H.

A



B

Spot	Protein	Mass	MASCOT Score	Peptides matched	Accession #
1	Apolipoprotein A-I	30759	931	20	37499465
2	CD5 antigen-like (scavenger receptor cystein rich family)	39603	347	10	11967471
3	Fibrinogen, gamma polypeptide	50092	1074	41	30583001
4	Albumin	71344	252	9	23307793
5	Complement factor H	143654	611	15	56203410

FIGURE 3. Five human plasma proteins that interact with TRX-1. Representative fluorescence-stained two-dimensional gels showing human plasma proteins bound to aC35S-TRX-1-coupled column (**A**, *left*) and after preincubation of human plasma with rhTRX-1 (**A**, *right*). The circled spots were reduced in intensity by preincubation with rhTRX-1. Five protein spots from two-dimensional gels identified by mass spectrometry and their NCBI accession numbers (**B**).

Regulation of Complement Activation by TRX-1

To confirm the interaction between TRX-1 and factor H, Western blotting was performed with human plasma to which TRX-1 had been added (Fig. 4A) and with mixtures of purified factor H and TRX-1 (Fig. 4B). In the mixtures of human plasma plus TRX-1, no changes were observed by silver staining (Fig. 4A). However, the anti–TRX-1 mAb labeled several bands, in addition to the TRX-1 band (12 kDa), especially when 1 μM TRX-1 was added. One of these bands had the same molecular weight as the single band labeled by the anti–factor H mAb in Western blots (Fig. 4A). In addition, careful superimposition of the blots showed that this band, detected by the anti–factor H mAb, shifted up in molecular weight at the highest dose (1 μM) of TRX-1. This increase in molecular weight was confirmed in blots using purified factor H (139 kDa), by silver staining, and anti–factor H mAb, and by anti–TRX-1 mAb, when more equimolar TRX-1 (1 and 10 μM) was added (Fig. 4B). Silver staining showed the apparent shift up of the bands when factor H was incubated with 1 and 10 μM TRX-1. Moreover, Western blotting using anti–factor H mAb showed that factor H was not recognized by this anti–factor H mAb when it was incubated with an excess amount (10 μM) of TRX-1. These provided further evidence of the interaction between TRX-1 and factor H.

Finally, we examined the effect of TRX-1 on the function of factor H, as a major regulator of the alternative complement pathway, inhibiting the conversion of C3 to C3a and C3b. Using a fluid-phase method (Fig. 4C),[21] the generation of C3a from C3 was detected by adding factor B and factor D and increasing concentrations of factor H to inhibit this enzyme reaction. Surprisingly, we found that TRX-1 alone inhibited C3 cleavage dose dependently and that its effect was additive when factor H was also present. No effect on C3 cleavage was seen when DM-TRX-1 was used. These results indicated that TRX-1 acts as a regulator of the complement pathway not only in association with factor H but also on its own and that its active site must function in this process.

DISCUSSION

The clinical diagnostic characteristic of AMD at an early stage is the appearance of drusen that deposit between RPE and Bruch membrane. Earlier studies demonstrate that the presence of complement component in drusen has the possibility of complement activation in AMD.[4,5] Moreover, polymorphisms in the CFH are associated with a significantly increased risk for the development of AMD.[6–9] Factor H is present abundantly in plasma (roughly 500 μg/mL) and is composed of 20 repetitive units of 60 amino acids, named

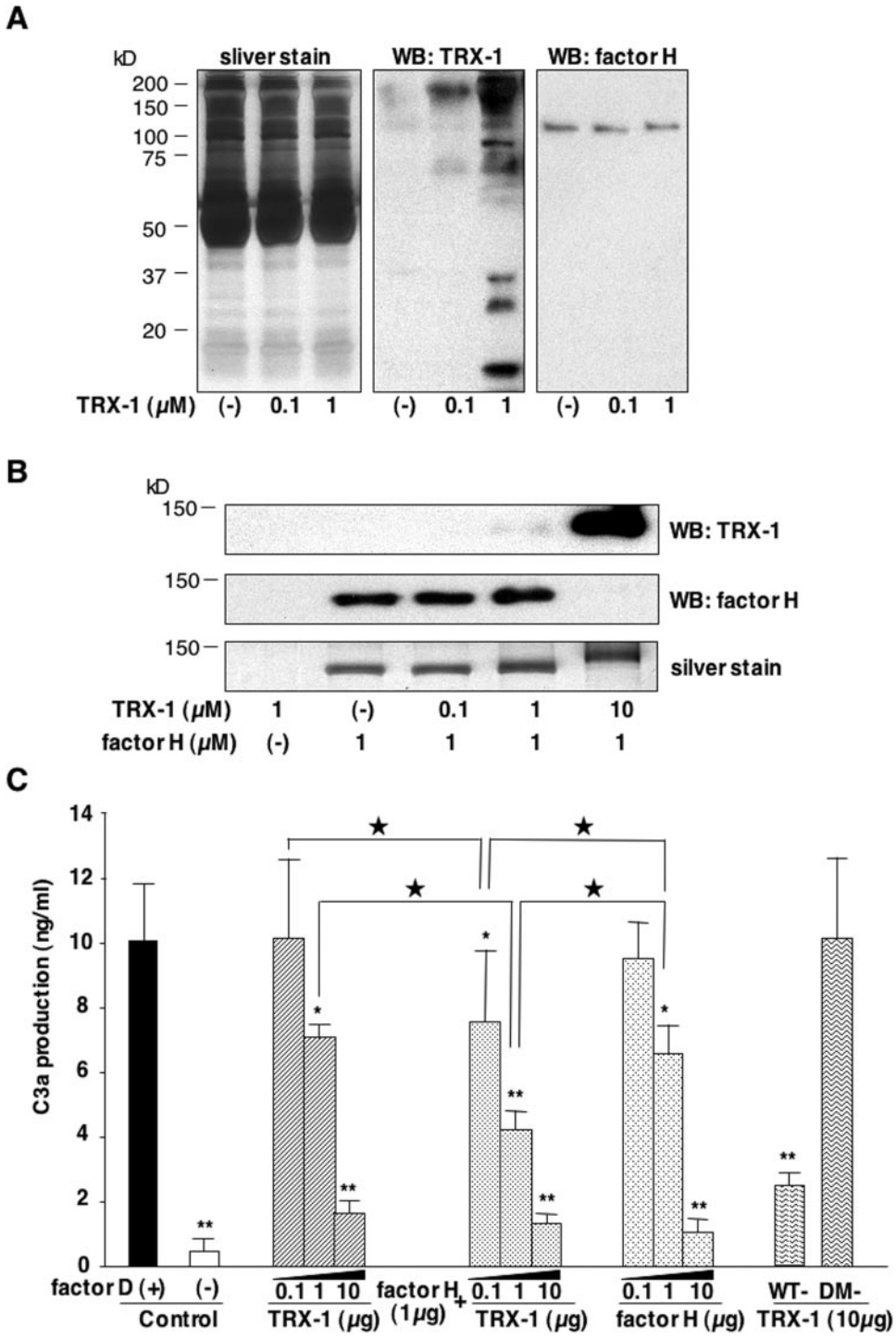


FIGURE 4. TRX-1 interacts with factor H and regulates complement activation. Silver staining and Western blot analysis showed TRX-1 and factor H in human serum plasma incubated with increasing concentrations of TRX-1 (**A**) and with the addition of purified factor H and various concentrations of TRX-1 (**B**). ELISA for C3a showing the inhibition of C3 convertase by TRX-1 and factor H, in mixtures of purified C3, C3i, factor B, and factor D in the presence of Mg^{++} (**C**). Data are mean ± SD ($n = 3$; $^{*}P < 0.05$; $^{**}P < 0.01$ vs. positive control; $\star P < 0.05$ TRX-1 or factor H alone vs. both).

the short consensus repeat (SCR) or the complement control protein module.[24,25] The SCRs include four cysteines that form two disulfide bonds in Cys1-Cys3 and Cys2-Cys4. Factor H has three C3b-binding sites in SCR1–4, SCR12–14, and SCR19–20, and the binding to C3b by factor H promotes the factor I–dependent inactivation of C3b. In this study, we confirmed that TRX-1 interacted with factor H and that TRX-1 acted additively to the function of factor H in the alternative complement pathway. These data suggest that the interacting site with TRX-1 may overlap with the C3b binding site in factor H. Moreover, the disulfide/dithiol exchange between TRX-1 and factor H may not interfere with the C3b binding of factor H. In fact, adrenomedullin also binds to factor H and enhances the complement regulatory function of factor H.[26] Factor H reciprocally enhances the function of adrenomedullin through their interaction. TRX-1 and factor H may have a similar interaction. Furthermore, we confirmed that TRX-1 regulated the alternative complement pathway. The alternative pathway convertase is formed by an interaction between C3b and factor B, followed by the cleavage of factor B by factor D into Ba and Bb, to form an active enzymatic complex, C3bBb. This conver-

tase is then able to generate more C3b molecules, initiating a positive feedback loop in which Ba consists of three SCRs. The present data suggested the disulfide/dithiol exchange between TRX-1 and SCRs of factor B may inhibit the production of C3bBb through the interaction between C3b and factor B or the cleavage of factor B by factor D.

Expression of TRX is reported in the nerve and photoreceptor cells in rat retina,[27] and is upregulated by oxidative stresses such as photooxidative stress.[28] TRX-1 Tg mice were C57BL/6 mice that carried the human TRX-1 transgene under the control of the β-actin promoter and expressed human TRX throughout the body, including the retina and RPE, in our previous studies.[17,29] The expression level of human TRX-1 at various tissues in TRX-1 Tg mice were roughly threefold to sixfold higher than those of mouse endogenous TRX-1 in wild-type mice.[17] We also investigated anti-inflammatory effects of TRX-1 in suppressing the activation of p38 MAPK, L-selectin shedding, the adhesion of neutrophils to endothelial cells and neutrophil extravasation.[14–16] However, the precise molecular mechanisms by which extracellular TRX-1 exerts these effects are not fully understood.

The complement system is a highly conserved component of the mammalian host defense system and has been intensively studied. The terminal complement cascade can be activated through three distinct pathways: the classical, alternative, and lectin pathways. Although all three pathways involve the activation of C3 convertase and the production of C3a and C3b, followed by the terminal C5b-9 complex, they are regulated independently.[30] We have demonstrated that the redox protein TRX-1 can itself activate C3 convertase. Factor H—which inhibits the conversion of C3 to C3a and C3b and inactivates C3b on cells and in the circulation—is recognized as the main regulator of this enzyme. This alternative pathway is critical in the development of CNV.[18] C5a, which is produced as a result of C3 activation, is a potent activator of neutrophils. Here we have shown that TRX-1 can suppress the activation of C3 and the infiltration of neutrophils into inflammatory sites. We previously reported that TRX-1 suppresses neutrophil infiltration by downregulating the expression of chemokines in autoimmune myocarditis in mice.[16] Therefore, we measured the inflammatory chemokines, such as macrophage inflammatory protein (MIP-2) and KC, by using an ELISA kit (R&D Systems, Minneapolis, MN) to confirm the further mechanism of neutrophil infiltration. However, the levels of MIP-2 and KC were not significantly different between wild-type and TRX-1 Tg mice 2 days after laser (MIP-2, 58.1 ± 17.8 ng/mL vs. 49.1 ± 14.7 ng/mL; $n = 6$; KC, 34.4 ± 14.9 ng/mL vs. 29.9 ± 8.8 ng/mL; $n = 6$). Our data suggest that the anti-inflammatory effects of TRX-1 can be explained, at least in part, by its ability to regulate the complement system. Although further studies will clarify the significance of TRX-1 as a redox regulator of the complement system in host defense, the results presented here suggest novel approaches to the development of new therapies for AMD.

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References

1. Tomany SC, Wang JJ, Van Leeuwen R, et al. Risk factors for incident age-related macular degeneration: pooled findings from 3 continents. *Ophthalmology*. 2004;111:1280–1287.
2. van Leeuwen R, Klaver CC, Vingerling JR, Hofman A, de Jong PT. Epidemiology of age-related maculopathy: a review. *Eur J Epidemiol*. 2003;18:845–854.
3. Ames BN, Shigenaga MK, Hagen TM. Oxidants, antioxidants, and the degenerative diseases of aging. *Proc Natl Acad Sci U S A*. 1993;90:7915–7922.
4. Nozaki M, Raisler BJ, Sakurai E, et al. Drusen complement components C3a and C5a promote choroidal neovascularization. *Proc Natl Acad Sci U S A*. 2006;103:2328–2333.
5. Bora PS, Sohn JH, Cruz JM, et al. Role of complement and complement membrane attack complex in laser-induced choroidal neovascularization. *J Immunol*. 2005;174:491–497.
6. Edwards AO, Ritter R 3rd, Abel KJ, et al. Complement factor H polymorphism and age-related macular degeneration. *Science*. 2005;308:421–424.
7. Haines JL, Hauser MA, Schmidt S, et al. Complement factor H variant increases the risk of age-related macular degeneration. *Science*. 2005;308:419–421.
8. Klein RJ, Zeiss C, Chew EY, et al. Complement factor H polymorphism in age-related macular degeneration. *Science*. 2005;308:385–389.
9. Gold B, Merriam JE, Zernant J, et al. Variation in factor B (BF) and complement component 2 (C2) genes is associated with age-related macular degeneration. *Nat Genet*. 2006;38:458–462.
10. Holmgren A. Thioredoxin. *Annu Rev Biochem*. 1985;54:237–271.
11. Nakamura H, Nakamura K, Yodoi J. Redox regulation of cellular activation. *Annu Rev Immunol*. 1997;15:351–369.
12. Saitoh M, Nishitoh H, Fujii M, et al. Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. *EMBO J*. 1998;17:2596–2606.
13. Nishiyama A, Matsui M, Iwata S, et al. Identification of thioredoxin-binding protein-2/vitamin D(3) up-regulated protein 1 as a negative regulator of thioredoxin function and expression. *J Biol Chem*. 1999;274:21645–21650.
14. Nakamura H, Herzenberg LA, Bai J, et al. Circulating thioredoxin suppresses lipopolysaccharide-induced neutrophil chemotaxis. *Proc Natl Acad Sci U S A*. 2001;98:15143–15148.
15. Hoshino T, Nakamura H, Okamoto M, et al. Redox-active protein thioredoxin prevents proinflammatory cytokine- or bleomycin-induced lung injury. *Am J Respir Crit Care Med*. 2003;168:1075–1083.
16. Liu W, Nakamura H, Shioji K, et al. Thioredoxin-1 ameliorates myosin-induced autoimmune myocarditis by suppressing chemokine expressions and leukocyte chemotaxis in mice. *Circulation*. 2004;110:1276–1283.
17. Takagi Y, Mitsui A, Nishiyama A, et al. Overexpression of thioredoxin in transgenic mice attenuates focal ischemic brain damage. *Proc Natl Acad Sci U S A*. 1999;96:4131–4136.
18. Bora NS, Kaliappan S, Jha P, et al. Complement activation via alternative pathway is critical in the development of laser-induced choroidal neovascularization: role of factor B and factor H. *J Immunol*. 2006;177:1872–1878.
19. Hirota K, Matsui M, Iwata S, et al. AP-1 transcriptional activity is regulated by a direct association between thioredoxin and Ref-1. *Proc Natl Acad Sci U S A*. 1997;94:3633–3638.
20. Kinumi T, Ogawa Y, Kimata J, et al. Proteomic characterization of oxidative dysfunction in human umbilical vein endothelial cells (HUVEC) induced by exposure to oxidized LDL. *Free Radic Res*. 2005;39:1335–1344.
21. McRae JL, Duthy TG, Griggs KM, et al. Human factor H-related protein 5 has cofactor activity, inhibits C3 convertase activity, binds heparin and C-reactive protein, and associates with lipoprotein. *J Immunol*. 2005;174:6250–6256.
22. Motohashi K, Kondoh A, Stumpp MT, Hisabori T. Comprehensive survey of proteins targeted by chloroplast thioredoxin. *Proc Natl Acad Sci U S A*. 2001;98:11224–11229.
23. Walport MJ. Complement: first of two parts. *N Engl J Med*. 2001;344:1058–1066.
24. Rodriguez de Cordoba S, Esparza-Gordillo J, Goicoechea de Jorge E, Lopez-Trascasa M, Sanchez-Corral P. The human complement factor H: functional roles, genetic variations and disease associations. *Mol Immunol*. 2004;41:355–367.

25. Kirkitadze MD, Barlow PN. Structure and flexibility of the multiple domain proteins that regulate complement activation. *Immunol Rev*. 2001;180:146–161.
26. Pio R, Martinez A, Unsworth EJ, et al. Complement factor H is a serum-binding protein for adrenomedullin, and the resulting complex modulates the bioactivities of both partners. *J Biol Chem*. 2001;276:12292–12300.
27. Hansson HA, Holmgren A, Norstedt G, Rozell B. Changes in the distribution of insulin-like growth factor I, thioredoxin, thioredoxin reductase and ribonucleotide reductase during the development of the retina. *Exp Eye Res*. 1989;48:411–420.
28. Tanito M, Masutani H, Kim YC, et al. Sulforaphane induces thioredoxin through the antioxidant-responsive element and attenuates retinal light damage in mice. *Invest Ophthalmol Vis Sci*. 2005;46:979–987.
29. Tanito M, Masutani H, Nakamura H, et al. Attenuation of retinal photooxidative damage in thioredoxin transgenic mice. *Neurosci Lett*. 2002;326:142–146.
30. Huber-Lang M, Sarma JV, Zetoune FS, et al. Generation of C5a in the absence of C3: a new complement activation pathway. *Nat Med*. 2006;12:682–687.