Published in final edited form as:

Invest Ophthalmol Vis Sci. 2009 June ; 50(6): 2896–2902. doi:10.1167/iovs.08-2495.

Biologically Active Fibronectin Fragments Stimulate Release of MCP-1 and Catabolic Cytokines from Murine Retinal Pigment Epithelium

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Abstract

Purpose—High-temperature requirement serine protease (*HTRA1*) was identified as a candidate age-related macular degeneration gene in multiple genetic studies in humans. To date, no functional studies have shown a mechanism for *HTRA1* to instigate ocular tissue abnormalities. In the present study, the authors focused on a substrate of *HTRA1*, fibronectin, because fibronectin fragments (Fnfs) stimulate biochemical events in other age-related degenerative diseases that are analogous to changes associated with age-related macular degeneration (AMD). The purpose of the study was to determine whether Fnfs stimulate the release of proinflammatory and catabolic cytokines from murine retinal pigment epithelium (RPE).

Methods—Fibronectin was purified from murine serum by gelatin cross-linked agarose chromatography and subsequently was enzymatically digested with α -chymotrypsin. The bioactivity of Fnfs was verified by measuring levels of IL-6 and TNF- α in Fnf-exposed murine splenocytes. To analyze the effect of Fnfs on RPE, cytokine and chemokine levels in RPE culture supernatants were assayed by ELISA.

Results—IL-6 and TNF- α proinflammatory cytokines were released from primary murine splenocytes in proportion to the dose and length of Fnf treatment, indicating that α -chymotryptic digests of fibronectin are biologically active. Fnf treatment of murine RPE cells stimulated the release of microgram and nanogram levels of IL-6, MMP-3, MMP-9, and MCP-1, whereas only picogram levels were detected in untreated cells.

Conclusions—Fnfs stimulate the release of proinflammatory cytokines, matrix metalloproteinases, and monocyte chemoattractant protein from murine RPE cells. This observation indicated that Fnfs could contribute to ocular abnormalities by promoting inflammation, catabolism, and monocyte chemoattraction.

Age-related macular degeneration (AMD) is the most common cause of irreversible visual impairment in patients older than 50.¹ As the baby boomer generation ages, the incidence of AMD in the United States is expected to increase to 3 million.² Despite knowledge about the clinical and pathologic features of AMD, little is known about its underlying cause or causes. Previous attempts to identify an underlying cause for AMD have included genetic studies. More specifically, multiple groups have screened humans with AMD for evidence of restriction fragment length polymorphisms.

High-temperature requirement serine protease (*HTRA1*) polymorphisms have been strongly associated with AMD in diverse human populations.³⁻³⁰ In human eyes taken from persons with AMD, *HTRA1* levels are upregulated in the macular region,⁶ yet no functional studies have mechanistically linked alterations in *HTRA1* expression with ocular changes. Substrates of *HTRA1* are extracellular matrix proteins, including fibronectin.

Fibronectin is present in Bruch's membrane, underlying the retinal pigment epithelium (RPE), where it anchors the RPE to the basal lamina through $\alpha 5\beta 1$ integrins.³¹ Fibronectin is more abundantly expressed by the RPE of donors with AMD than of age-matched controls,³² and fibronectin accumulates in basal linear deposits in the macula of elderly eyes.³³ Furthermore, fibronectin is abundant in drusen,³⁴ defined as the abnormal accumulation of extracellular deposits in the basal aspect of the RPE, which is a risk factor for AMD.^{35,36} We were intrigued by the observation that recombinant *HTRA1* can directly digest fibronectin to produce fibronectin fragments (Fnfs)^{37,38} and reasoned that the Fnfs could be a downstream protagonist of intraocular inflammation and catabolism.

Fnfs have been studied extensively in age-related inflammatory diseases outside the eye. It is established that intact fibronectin is capable of binding to many different types of receptors on a diverse range of cells, including fibroblasts, epithelium, endothelium, neural crest, B and T lymphocytes, monocytes, and megakaryocytes. Furthermore, it is known that Fnfs are more potent stimulators of proinflammatory and catabolic cytokines than intact fibronectin. One of the best-studied inflammatory diseases of aging, in which Fnfs play a central role, is arthritis. Two well-studied examples are osteoarthritis and rheumatoid arthritis. Specifically, Fnfs accumulate in synovial fluid in arthritic joints.³⁹⁻⁴³ The accumulation of Fnfs causes pathology through multiple biochemical mechanisms, including increased release of matrix metalloproteinases⁴⁴⁻⁴⁹ and enhanced accumulation of proinflammatory cytokines, among them IL-6 and TNF- α .^{33,44,50,51} Given that AMD is also associated with increased levels of matrix metalloproteinases (MMPs) and proinflammatory cytokines, Fnfs may contribute to these changes.

Investigators have recently started to consider that drusen accumulation may be secondary to RPE abnormalities involving chronic inflammation.⁵²⁻⁵⁷ Therefore, we designed our study to examine the impact of Fnfs on the secretion of proinflammatory and catabolic cytokines from the RPE by testing the hypothesis that exposure of murine RPE cells to high levels of Fnfs stimulates the RPE to release catabolic and proinflammatory cytokines. We show that Fnf treatment of murine RPE cells causes enhanced secretion of IL-6, MCP-1, MMP-3, and MMP-9.

Methods

Primary Cell Culture

To establish primary cell cultures, C57BL/6 mice (age range, 3-6 months) were euthanatized by CO₂ asphyxiation. Animal care was conducted in compliance with National Institutes of Health guidelines and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Unless specified otherwise, culture reagents were from Invitrogen/Gibco (Carlsbad, CA). Primary cell cultures were incubated at 37°C in 5% CO₂.

Splenocytes

Spleens were removed from C57BL/6 mice after euthanatization. The connective tissue capsule was removed, and cells were gently suspended in medium with a Pasteur pipette. The cell suspension was pushed through a 70- μ M cell strainer (BD Biosciences, Bedford, MA). Cells were clarified by centrifugation (300 rcf [relative centrifugal force] $\times$ 5 minutes). Pelleted cells

were resuspended in lysing buffer (Ack; Quality Biological, Inc., Gaithersburg, MD) and incubated for 5 minutes at room temperature. Cells were washed in 10 vol medium. Then cells were plated (1×10^6 cells/mL in a 24-well plate) in RPMI supplemented with 10% fetal bovine serum, 1% glutamine, and a 1% antibiotic solution containing penicillin, streptomycin, and glutamine (catalog no. 10378-016; Gibco).

Retinal Pigment Epithelium

Eyes were removed from C57BL/6 mice and transferred to a Petri dish containing supplemented RPMI (10% fetal bovine serum, 1% glutamine, and a 1% antibiotic solution containing penicillin, streptomycin, and glutamine [catalog no. 10378-016; Gibco], and $1 \times N1$ medium supplement for neural cell cultures [N6530; Sigma, St. Louis, MO]). Corneas, lenses, vitreous, and retinas were dissected away from the posterior globe. RPE cells were loosened from the posterior eyecup by digestion in 0.25% trypsin-EDTA (30-40 minutes at 37°C). Cells were later suspended in medium by triturating complete medium in the digested posterior globe. Cells were then washed once and were plated (60,000-100,000 cells/well) in a 96-well plate.

Fibronectin Fragments

Fibronectin was purified from normal mouse serum (Biodesign International, Saco, ME) by binding to gelatin cross-linked agarose (Sephacrose 4B; Amersham Biosciences, Piscataway, NJ) and subsequent elution with 4 M urea. Protein elution was monitored by measuring spectrophotometric absorbance at 280 nm. Eluted protein was dialyzed against $1 \times$ PBS. Purified fibronectin was digested overnight at 37°C with the following enzymes: 1 U/mL thrombin, 0.3 U/mL cathepsin-D, and 1 U/mL α -chymotrypsin (all from Calbiochem, La Jolla, CA). Because α -chymotrypsin provided the most effective enzyme digestion (Fig. 1A), it was used in later studies. Overnight digestion with α -chymotrypsin was found to be too long and did not produce a broad spectrum of fragments (Fig. 1A); therefore, time-course enzymatic digestion was subsequently performed with α -chymotrypsin to optimize the methodology. Ten-minute digestions were found to be optimal (Fig. 1B).

SDS-PAGE

Digestion of fibronectin was verified by electrophoresis into 4%-20% Bis-Tris gels (Invitrogen, Carlsbad, CA), followed by staining (SimplyBlue SafeStain; Invitrogen). Parallel Western blot detection of electrophoresed Fnfs, with rabbit anti-fibronectin antibodies (AbD Serotec, Raleigh, NC), produced the same band pattern as the stained gels (not shown).

Measurement of Cytokine Levels

IL-6 and TNF- α in splenocyte cell culture supernatants were analyzed with commercially available ELISA in accordance with the manufacturer's instructions (BD Biosciences, San Jose, CA). Supernatants from primary murine RPE cultures were submitted to ThermoFisher Scientific (Woburn, MA) for cytokine testing services (SearchLight; Pierce, Rockford, IL).

Statistical Analysis

Data were analyzed and graphed (GraphPad Prism 5; La Jolla, CA). Specifically, either *t*-tests or Kruskal-Wallis ANOVA, followed by Dunn's multiple comparison tests to identify differences among groups, were performed.

Results

Fnfs Prepared by α -Chymotrypsin Digestion Are Biologically Active

Fibronectin is a dimeric protein composed of two subunits (220-250 kDa). Because thrombin, cathepsin-D, and α -chymotrypsin have previously been used to produce biologically active

Fnfs, gelatin-purified murine serum fibronectin was digested with each enzyme (Fig. 1A). In our hands, digestion of murine fibronectin with α -chymotrypsin was more efficient than digestion with thrombin or cathepsin-D (Fig. 1A). Time-course enzymatic digestion was subsequently performed with α -chymotrypsin to optimize the methodology. The 10-minute digestion produced 11 distinct Fnfs (Fig. 1B). The same pattern of Fnfs shown in the representative image (Fig. 1B) was found in three separate 10-minute α -chymotrypsin enzyme digests, performed at different times (not shown).

To verify that the Fnfs were biologically active, murine splenocytes were treated with Fnfs for 1 to 24 hours. Levels of IL-6 and TNF- α were used as indicators of Fnf bioactivity. IL-6 and TNF- α in splenocytes exposed to Fnfs increased with time (Figs. 2A, B), whereas only basal levels of expression were found in splenocytes treated with vehicle (PBS) or enzyme alone (boiled α -chymotrypsin). Splenocytes exposed to the protease inhibitor used to stop the fibronectin digestion did not release significantly different levels of IL-6 or TNF- α compared with vehicle (not shown).

Murine RPE Cells Exposed to Fnfs Release Proinflammatory and Catabolic Cytokines and MCP-1

Proinflammatory Cytokines—Very low levels of IL-6 were detected in supernatants from murine RPE cells exposed to vehicle or enzyme only (Fig. 3A). Murine RPE cells exposed to low-dose Fnfs (10 μ g) secreted more IL-6 than vehicle or enzyme-only treated cells, but the levels did not reach significance (Fig. 3A). Significantly more IL-6 was detected in supernatants from murine RPE cells exposed to the highest dose of Fnfs (50 μ g) than from vehicle or enzyme-only treatments (Fig. 3A). Fnfs did not induce the secretion of IL-6 from murine RPE cells when the Fnfs were first boiled for 10 minutes (Fig. 3A). We also verified the observed differences in Figure 3A by standard ELISA and found significant differences ($P < 0.05$) for IL-6 levels (not shown). TNF- α levels also increased in response to the amount of Fnf treatment; however, the levels were not significantly different from vehicle or enzyme-only treatments (Fig. 3B).

Levels of anti-inflammatory cytokines (IL-4 and IL-13) were also analyzed but were not found to be significant (not shown). IL-4 levels on day 3 were as follows: vehicle, 6.6 ± 3.6 pg/mL; 10 μ g Fnf, 9 ± 2 pg/mL; 50 μ g Fnf, 0 ± 0 pg/mL. IL-13 levels on day 3 were as follows: vehicle, 31.3 ± 11.5 pg/mL; 10 μ g Fnf, 101.4 ± 4.6 pg/mL; 50 μ g Fnf, 125.2 ± 29.8 pg/mL.

MCP-1—Levels of MCP-1 in RPE culture supernatants from cells treated with 10 and 50 μ g Fnf were significantly higher the day after treatment than were supernatants from cells treated with enzyme only or vehicle (Fig. 4). After 3 days, cells exposed to 50 μ g Fnf secreted significantly more MCP-1 than all other treatment groups (Fig. 4). The effectiveness of Fnfs to induce the secretion of MCP-1 was lost when the Fnfs were boiled before the RPE cells were treated (Fig. 4). Although the levels of MCP-1 detected by antibody array were variable, mean values changed by more than 100-fold when 50- μ g Fnf treatment was compared with vehicle treatment (Fig. 4).

Catabolic Cytokines—To determine whether Fnfs are capable of increasing catabolism, we first analyzed the ability of supernatants from Fnf-treated primary murine RPE cells to digest fibronectin, casein, and gelatin (not shown). Enzymography using each substrate showed enhanced activation of MMP activity with respect to the dose of Fnf treatment (not shown). Based on molecular weight and substrate digestion, we decided to further examine levels of MMP-2, MMP-3, and MMP-9 in RPE culture supernatants by ELISA.

Fnf treatment of murine RPE cells did not affect the secretion of MMP-2 (Fig. 5A). MMP-3 levels were significantly elevated in RPE cells exposed to 50 μ g Fnf for 3 days (Fig. 5B)

compared with all other treatment groups. The effectiveness of Fnfs to induce MMP-3 secretion from murine RPE cells was lost when the Fnfs were boiled (Fig 5B). MMP-9 levels were significantly elevated in murine RPE culture supernatants from cells exposed to 50 μ g Fnf after 1 day and 3 days compared with other treatment groups (Fig. 5C). The effectiveness of Fnfs to induce MMP-9 secretion from murine RPE cells was lost when the Fnfs were boiled (Fig. 5C).

Discussion

The RPE is a monolayer of cuboidal cells located between the retina and the underlying choroidal vasculature. Proper functioning of the RPE is critical to the maintenance of ocular homeostasis and vision because the RPE has diverse functions, including phagocytosis of rods and cones, processing of vitamin A, and limiting transport of materials from the choroid to the retina.⁵⁸ The dysfunction of RPE is implicated in AMD. Although changes in photoreceptors, RPE, Bruch's membrane, and choroid are all seen in human patients with AMD, changes in RPE function are thought to be early events in the mechanism leading to the development of clinical AMD changes that cause photoreceptor loss.^{36,59}

Inflammatory mediators and inflammatory cells are increasingly implicated in the progression of AMD,^{53,60} and the role of the RPE in determining whether the eye has a downimmuno-regulatory environment becomes increasingly more important over time.^{54,61} It has been suggested that a chronic inflammatory environment in the eye can lead to drusen biogenesis,^{57,62} one of the clinically visible signs associated with AMD progression. Similarly, drusen are thought to be biomarkers of an immune-mediated process at the RPE-Bruch's membrane interface.⁵⁵

In our study, we found that Fnf treatment of RPE cells enhanced the secretion of proinflammatory cytokines (Fig. 3), whereas differences in anti-inflammatory cytokine (IL-4, IL-13) secretion did not reach significance (data not shown). IL-6 release from RPE cells is an important observation because IL-6 has previously been shown to enhance choroidal neovascularization (CNV) through the induction of STAT3, whereas antibody neutralization of IL-6 suppressed CNV and resulted in reduced secretion of MCP-1 and macrophage infiltration into the area of CNV in a mouse model of laser-induced CNV.⁶³ We were unable to show that Fnf treatment leads to enhanced Stat3 phosphorylation in murine RPE cells because it was difficult to obtain enough cells to detect a change. However, we did find that Fnfs affect Stat3 phosphorylation in human peripheral blood mononuclear cells, but the results were highly variable for different donors (data not shown).

Along with proinflammatory cytokines, we also found that Fnfs greatly enhanced the release of MCP-1 (also known as CCL2) from the murine RPE. MCP-1 is known to be constitutively released from the RPE and is enhanced by proinflammatory cytokines.^{64,65} It is theoretically possible that this enhanced release of MCP-1 could attract resident ocular macrophages (microglia) and could contribute to digestion of the RPE and Bruch's membrane. Evidence is found in histologic specimens from humans with AMD, in whom macrophages are physically located in areas of RPE atrophy, Bruch's membrane breakdown, and choroidal neovascularization.⁶⁶⁻⁷⁰ In addition to affecting the RPE and the Bruch's membrane, such high levels of MCP-1 could negatively impact the retina because retinal detachment-induced photoreceptor apoptosis has been associated with MCP-1.⁷¹

Another way in which Fnfs can contribute to retinal degeneration is through the induction of MMPs. Matrix metalloproteinases are involved in normal tissue remodeling and repair. However, the dysregulation of MMP levels is associated with macular degenerative changes, such as CNV and basal laminar deposits in humans.⁷²⁻⁷⁵ MMP-3/stromelysin and MMP-2 and

MMP-9 (gelatinases A and B) are localized to the ventral aspect of the RPE in the interphotoreceptor matrix and vitreous.⁷⁶ Only the gelatinases are located in the basal aspect of RPE (in basal laminar deposits)⁷² and in CNV membranes.^{72,73,75} Our observation that Fnfs induced increased levels of MMP-3 secretion from the RPE is potentially relevant to an ongoing disease process. One substrate of MMP-3 is fibronectin; hence, it is conceivable that increased levels of Fnfs could serve as a protagonist of a chronic catabolic condition in the eye by which Fnfs initiate a positive feedback pathway for the release of catabolic cytokines from the RPE. Fnfs increase the release of MMP-3, which can act back on fibronectin to promote more Fnf accumulation.

Of all our observations, the ability of Fnfs to stimulate such high levels of MCP-1 warrants further investigation. Similarly, though fibronectin is known to accumulate in diffuse drusen (based on immunohistochemical staining),³⁴ there is no easy way to determine whether the fibronectin is fragmented and serves as a nidus for inflammation and catabolism in ocular tissue sections. Nevertheless, the present study is the first in which the role of Fnfs has been studied in ocular tissues. The ability of Fnfs to stimulate the secretion of such high levels of IL-6, MCP-1, and MMPs from the RPE underscores the importance of Fnfs in modulating acute inflammation in the eye. Their role in chronic ocular disease remains to be determined, and a method to stimulate the long-term accumulation of Fnfs in the eye remains to be developed.

Acknowledgments

Supported by a National Eye Institute Intramural Research Training Award.

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Figure 1.

Optimal enzymatic digestion of fibronectin, based on number of fragments, lasted 10 minutes with α -chymotrypsin. Stained gels of electrophoresed Fnfs are depicted. After enzymatic digestion, protease inhibitor (pi) was added. (A) Murine serum fibronectin was digested overnight with enzyme in 1 $\times$ PBS. *Lane 1*: undigested serum fibronectin + pi; *lane 2*: fibronectin digested with 1 U/mL thrombin + pi; *lane 3*: fibronectin digested with 0.3 U/mL cathepsin-D + pi; *lane 4*: fibronectin digested with 1 U/mL α -chymotrypsin + pi. (B) Time-course enzymatic digestion with 1 U/mL α -chymotrypsin. *Lane 1*: serum fibronectin undigested + pi; *lane 2*: 10-minute digestion + pi; *lane 3*: 30-minute digestion + pi; *lane 4*: 1-hour digestion + pi; *lane 5*: 3-hour digestion + pi; *lane 6*: serum fibronectin only (boiled); *lane 7*: enzyme only (boiled); *lane 8*: pi only.

Figure 2.

Splenocytes exposed to Fnfs secrete IL-6 and TNF- α in a time- and dose-dependent fashion, indicating that Fnfs prepared by α -chymotrypsin digestion are biologically active. Murine splenocytes (1×10^6) were treated with heat-inactivated α -chymotrypsin (Δ enz), PBS (vehicle), or 10 μ g Fnf (Fnf) for 1 to 24 hours; $n = 3$ (one experiment) (**A**, **B**). The 24-hour time point was chosen for analysis of cytokine secretion in murine splenocytes (1×10^6) treated with 10 or 100 μ g Fnf or PBS (vehicle); $n = 6$ (two experiments). IL-6 and TNF- α were analyzed in culture supernatants using a commercially available ELISA. Data (**C**, **D**) were analyzed with the use of two-tailed t -tests for vehicle versus Fnf treatment groups (*** $P \leq 0.001$). Data were plotted as mean $\pm$ SEM.

Figure 3.

Murine RPE cells exposed to Fnfs secrete IL-6. C57BL/6 primary murine RPE cells (60,000-100,000) were plated in 96-well plates. Cells were exposed to heat-inactivated enzyme (Δ enz), vehicle (serum-free media), 10 μ g Fnf, or 50 μ g Fnf. Supernatants were collected on day 1 and day 3, and levels of IL-6 (**A**) and TNF- α (**B**) were analyzed with multiplex antibody arrays. Data were analyzed with the use of non-parametric Kruskal-Wallis tests, followed by Dunn's multiple comparison tests, to identify differences among groups (* $P < 0.05$). Data were plotted as mean $\pm$ SEM.

Figure 4.

Murine RPE cells exposed to Fnfs secrete MCP-1. C57BL/6 primary murine RPE cells (60,000-100,000) were plated in 96-well plates. Cells were exposed to heat-inactivated enzyme (Δ enz), vehicle (serum-free media), 10 μ g Fnf, or 50 μ g Fnf. Supernatants were collected on day 1 and day 3, and levels of MCP-1 were analyzed with multiplex antibody arrays. Data were analyzed with the use of nonparametric Kruskal-Wallis tests, followed by Dunn's multiple comparison tests, to identify differences among groups (* $P < 0.05$). Data were plotted as mean $\pm$ SEM.

Figure 5.

Murine RPE cells exposed to Fnfs secrete MMP-3 and MMP-9. C57BL/6 primary murine RPE cells (60,000-100,000) were plated in 96-well plates. Cells were exposed to heat-inactivated enzyme (Δ enz), vehicle (serum-free media), 10 μ g Fnf, or 50 μ g Fnf. Supernatants were collected on day 1 and day 3, and levels of MMP-2 (A), MMP-3 (B), and MMP-9 (C) were analyzed with multiplex antibody arrays. Data were analyzed with the use of nonparametric Kruskal-Wallis tests, followed by Dunn's multiple comparison tests, to identify differences among groups (* $P < 0.05$; ** $P < 0.01$). Data were plotted as mean $\pm$ SEM.