Inhibitory effect of an antibody to cryptic collagen type IV epitopes on choroidal neovascularization

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Purpose: The wet form of age-related macular degeneration (AMD) occurs as a consequence of abnormal blood vessel growth from the choroid into the retina. Pathological angiogenesis during tumor growth and ocular disease has been associated with specific exposure of cryptic extracellular matrix epitopes. We investigated the presence of collagen IV epitopes in a murine model of choroidal neovascularization (CNV), and tested the effect on blood vessel growth of H8, a humanized antibody directed against a cryptic collagen type IV epitope.

Methods: To induce experimental CNV in adult C57BL/6 mice, Bruch’s membrane was ruptured using a diode laser. Subsequently, mice were treated with daily intraperitoneal (i.p.) injections of either H8 (10 mg/kg or 30 mg/kg) or an isotype-matched antibody control. Two weeks postinjection, choroidal flat mounts were immunostained with the blood vessel marker platelet/endothelial cell adhesion molecule-1 (PECAM-1) and H8. CNV was visualized using fluorescence microscopy and the CNV lesion area measured using Open Lab software.

Results: Collagen type IV and the cryptic epitope were observed at the site of laser-induced lesions. Staining with H8 was first observed three days post injury, two days after MMP2 expression in CNV lesions, becoming most intense five days following laser injury and extending beyond the area of neovascularization. At 14 days post injury, H8 staining was reduced in intensity, colocalized with the area of CNV, and was nearly absent from the underlying choroidal vessels. In addition, mice treated with H8 had a significant dose-dependent decrease in the area of CNV as compared to isotype-matched antibody controls.

Conclusions: Results suggest that exposure of cryptic collagen type IV epitopes is associated with the incidence of CNV and that the humanized antibody H8 may provide a new treatment for CNV.

Age-related macular degeneration (AMD) due to choroidal neovascularization (CNV) is the leading cause of blindness among the elderly in industrialized countries. Clinically, laser therapy and photodynamic therapy [1,2], irradiation [3], surgical removal [4], and macular translocation [5] are widely used to treat AMD. These therapies have limited application and are not without complications [6], therefore, a better understanding of the molecular mechanisms of CNV and new therapies for AMD are needed. Recently, anti-VEGF therapies (Macugen® and Lucentis®) targeting the abnormal neovascularization associated with AMD have been approved for treatment. Although they represent a breakthrough in the field, much remains to be understood about AMD, such as earlier detection and prevention of the disease and disease progression.

The extracellular matrix (ECM) is a molecular scaffold that not only provides mechanical support for tissues, but also regulates biochemical and cellular processes such as adhesion, migration, gene expression, and differentiation [7-11]. One of the most abundantly expressed ECM protein is collagen [12,13]. Collagen is composed of three amino acid chains organized in a triple helix and can be cleaved by a family of proteolytic enzymes termed matrix metalloproteinases (MMPs) [14,15]. While cellular interactions with intact triple helical collagen are known to regulate cell behavior via engagement of specific integrin receptors, proteolytic remodeling of collagen is also thought to be of great importance in the regulation of a number of disease processes, including tumor growth and angiogenesis [16-18]. In fact, protease antagonists inhibit neovascularization in animal models of CNV [19-21], suggesting that proteolytic remodeling of the ECM plays an important role in CNV.

Recently, Brooks and colleagues generated monoclonal antibodies directed against cryptic matrix epitopes that are exposed following proteolysis of collagen [22]. Studies using one of these antibodies, HUIV26, showed that proteolytic cleavage of collagen type IV, a major component of the vascular basement membrane [23], can expose a cryptic site that is associated with a shift in integrin binding [24] and that is normally hidden within the triple helical structure of collagen. Importantly, systemic administration of HUIV26 potently inhibited tumor-associated angiogenesis [24] and retinal neovascularization [25]. H8 is a humanized monoclonal antibody derived from HUIV26, also characterized for its recognition of cryptic epitopes in collagen IV following denaturation. Although it is clear that cryptic extracellular matrix epitopes are specifically exposed following proteolysis and play a functional role during angiogenesis, the participation
of cryptic ECM epitopes in CNV has not been explored. In the present study, we investigated the presence of cryptic ECM epitopes in CNV and tested the effect H8 on lesion size in a murine model of CNV.

METHODS

Animals: Male C57BL/6 mice weighing between 19 and 21 g were obtained from Charles River (Wilmington, MA). All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Mice were anesthetized with intramuscular ketamine hydrochloride (25 mg/kg) and xylazine (10 mg/kg) and their pupils dilated with 1% tropicamide. The mice were euthanized by intraperitoneal (i.p.) injection of a ketamine and xylazine overdose.

Antibodies and Reagents: H8 humanized IgG antibody and mouse monoclonal HUIV26 raised against denatured collagen type IV were supplied by Cancer Vax (Carlsbad, CA). Both triple-helical and denatured collagen type IV was detected using the polyclonal antibody AB769 (Chemicon, Temecula, CA). Endothelial cells were visualized with an antibody for platelet-endothelial cell adhesion molecule-1 (PECAM-1, clone MEC13.3; BD Biosciences Pharmingen, San Diego, CA) or biotinylated Griffonia simplicifolia lectin B4 (Vector Laboratories, Burlingame, CA). Secondary antibodies were conjugated to Alexa 488, Alexa 546 (Molecular Probes, Eugene, OR), or fluorescein (FITC; Jackson ImmunoResearch, West Grove, PA). Purified matrix metaloproteinase-2 was obtained from Chemicon. Thermal

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Figure 1. H8 binds to denatured collagen IV. A: ELISA showing that H8 preferentially recognizes heat denatured collagen IV. B: Binding specificity of HUIV26, the antibody from which H8 was derived. C: The cryptic collagen type IV site recognized by H8 is revealed by MMP-2 induced proteolysis. Murine retinal sections were incubated for 2 h with either control buffer or MMP-2 (1.0 µg/ml). Subsequently, sections were stained with H8 (green) and lectin (red). H8 staining in control (arrowhead) and MMP-2 treated samples (arrow). In the image, INL identifies the inner nuclear layer of the retina, ONL marks the outer nuclear layer of the retina; and CH indicates the choroid. The scale bar in the H8, Lectin, and Merge panels is equal to 50 µm. Human IgG (green) was used as an isotype-matched irrelevant antibody control and the scale bar is equal to 100 µm.
Laser Induced CNV: CNV was generated by modification of a previously described technique [26]. Four burns were created using diode laser photocoagulation (75 μm spot size, 0.1 s duration, 90 mW, Oculight SL laser; IRIDEX, Mountain View, CA) and a hand-held cover slide as a contact lens. Burns were localized to the 3, 6, 9, and 12 o’clock positions of the posterior pole of the retina. Production of a bubble at the time of laser photocoagulation, which indicates rupture of Bruch’s membrane, is an important factor in successfully inducing CNV. Therefore, only mice in which a bubble was produced for all four burns were included in the study. Subsequently, mice were treated with daily i.p. injections of 10 mg/kg (n=10) or 30 mg/kg (n=10) of H8 or an isotype-matched control antibody (n=12).

Quantification of CNV Lesions: Two weeks after laser injury, the size of CNV lesions was measured in choroidal flat-mounts. Following enucleation of the eye, choroidal flat-mounts were generated by removing the cornea and the lens, and then peeling the neural retina away from the underlying retinal pigment epithelium (RPE). Several radial cuts allowed the eye cup to be laid flat. Choroidal flat-mounts were fixed in 4% paraformaldehyde for 1 h at 4°C and stained using an antibody to PECAM-1 as described below. Flat-mounts were examined using fluorescence microscopy (DMRA2; Leica, Deerfield, IL). The area of CNV was defined as the total area of hyper-fluorescence associated with each burn, measured using Openlab imaging software (Improvision, Lexington, MA).

Immunofluorescence analysis: We used antibodies for pan-collagen type IV (2 μg/ml), H8 (10 μg/ml), and PECAM-1 (2 μg/ml) to visualize native, denatured collagen type IV and blood vessels, respectively, in CNV lesions. Eyes were enucleated and fixed with 4% paraformaldehyde for 1 h at 4°C. Choroidal flat-mounts were washed with phosphate-buffered saline (PBS), and placed in methanol for 20 min. Tissues were incubated overnight at 4°C with a primary antibody diluted in PBS containing 10% goat serum and 1% Triton X-100. Four washes in PBS were followed by incubation with a secondary antibody overnight at 4°C. Tissues were mounted using Vectashield mounting medium (Vector Laboratories) and examined by fluorescence microscopy.

For tissue sectioning, eyes were embedded in OCT (Sakura Finetek, Torrance, CA) and snap frozen in liquid nitrogen. For MMP-2-induced proteolysis, 10 μm frozen sections were incubated with control buffer alone (50 mM Tris, 200 mM NaCl, 10 mM CaCl2, pH 7.5) or with MMP-2 (1.0 μg/ml) for 2 h at 37°C. Sections were then washed and fixed in 4% PFA for 15 min. Subsequently, sections were incubated with primary antibody H8 (2 μg/ml) diluted in PBS containing 10% goat serum for 1 h at room temperature. After washing with PBS, sections were incubated with FITC-conjugated secondary antibody for 1 h at room temperature.

Statistical Analysis: All values are expressed as mean±SEM. ANOVA followed by a post hoc Bonferroni test was used to determine the significance of differences in multiple comparisons. Differences with a value of p<0.05 were considered significant.

RESULTS

H8 recognizes cryptic collagen type IV epitopes exposed by thermal denaturation and by MMP-2 induced proteolysis: We first tested whether H8, like its parent antibody HUIV26, recognizes collagen IV specifically. H8 preferentially recognizes thermally denatured collagen IV (Figure 1A) similarly to HUIV26 (Figure 1B). Previous studies have shown that HUIV26 recognizes cryptic collagen type IV epitopes exposed in the basement membrane of blood vessels following MMP induced proteolysis. We tested whether H8 could recognize epitopes revealed by MMP-2 induced proteolysis of collagen type IV. Non-fixed murine eye sections were incubated for 2 h with either control buffer or MMP-2 and subsequently stained with H8 and lectin. As shown in Figure 1C, sections treated with control buffer presented very weak H8 staining in retinal vessels and Bruch’s membrane. This weak staining appears to

Figure 2. H8 staining localizes to areas of CNV. A: There is no significant H8 staining present in the retina of the uninjured eye. B: Frozen sections of 7 day old CNV lesions incubated with either an IgG control antibody or with H8. The scale bar is equal to 25 μm.
be specific as it is not observed in samples incubated with an isotype matched irrelevant antibody (HuIgG), indicating a low level cross-reactivity of H8 with the native collagen IV surrounding retinal vessels and in Bruch’s membrane. In contrast, retinal and choroidal vessels in sections treated with MMP-2 were strongly positive for H8.

Presence of cryptic ECM epitopes on CNV: To detect the presence of denatured collagen type IV in CNV lesions, H8 immunohistochemistry was performed on frozen sections of eyes harvested 7 days after laser injury using uninjured eyes as controls. H8 labeled normal retinal vessels weakly (Figure 2A) but strongly labeled CNV lesions and the surrounding area (Figure 2B). The H8 staining observed in the inner retina neighboring the lesion site may be due to the exposure of cryptic collagen IV sites by the upregulation of proteases as a result of laser treatment. Isotype-matched antibody controls showed background fluorescence in the lesion site that was much lower in intensity than that observed for H8 staining.

Time course of cryptic epitope exposure following laser injury: Based on previous studies, we hypothesized that exposure of the cryptic collagen type IV epitope recognized by H8 may occur in the initial stages of CNV. Also, if ECM remodeling drives angiogenesis, we speculated that H8 staining should precede neovessel growth and that proteases implicated in ECM remodeling would precede H8 expression. To examine this possibility, we analyzed H8, MMP-2, MMP-9, and PECAM-1 expression during a time course of CNV development. We chose MMP-2 and MMP-9 as they have been associated with ECM remodeling leading to angiogenic growth [24,25]. Within an hour of laser treatment, a rim of H8 stain-

A

H8

MMP2

Merge

D1

D3

B

H8

PECAM

Merge

D5

D9

D14

C

H8

Collagen IV

Merge

D9

Figure 3. Time-course of expression of collagen type IV cryptic epitopes following Bruch’s membrane rupture by laser photocoagulation. A: Early CNV samples were costained with H8 (green) and MMP-2 (red) at days 1 and 3 following injury (D1, D3). MMP-2 staining precedes H8 at D1 and colocalizes with denatured collagen IV at D3. Scale bar=80 µm. B: Choroidal flat-mounts obtained at 5, 9, and 14 days (D5, D9, D14) after laser injury and costained with H8 (green) and PECAM-1 (red). H8 staining precedes neovessel growth at day 5 (D5) and colocalizes with neovessels by day 14 (D14). C: Choroidal flat mounts obtained at day 9 (D9) after laser injury were costained with H8 (green) and a collagen type IV antibody (red). Collagen type IV staining of the CNV lesion marked a larger area than did H8 staining. Scale bar=40 µm.

B

Control

H8

10 mg

30 mg

A

Control

H8

10 mg

30 mg

B

Control

H8 (10 mg)

H8 (30 mg)

Figure 4. H8 suppressed the development of CNV lesions at sites of laser induced rupture of Bruch’s membrane. Choroidal flat-mounts were examined by fluorescence microscopy following immunostaining with PECAM-1 to identify vessels. A: Animals treated with control antibody showed large areas of CNV as compared to animals treated with either 10 or 30 mg/kg of H8. B: Quantitation of CNV lesion size in IgG and H8 treated samples. Both 10 mg/kg and 30 mg/kg treatments caused a statistically significant reduction in lesion size as compared to control untreated animals (the asterisk indicates a p<0.05 and the double asterisk denotes a p<0.001). Scale bar=20 µm.
ing was observed around the lesion site, likely the outcome of photocoagulation (data not shown). MMP-2 staining first appeared at 1 day following injury (D1; Figure 3A), H8 expression was first observed at day 3 and colocalized with MMP-2 staining (D3; Figure 1A). We observed no MMP-9 expression at the lesion site (data not shown). The fluorescence intensity and area of staining for H8 were maximal at 5 days post laser burn and extended beyond the area of neovascularization (Figure 3B). At 9 days post injury, H8 staining remained strong and the area of neovascularization had grown to encompass the area stained by H8 (Figure 3B). At 14 days post injury, H8 staining was confined to the area of CNV as demonstrated by colocalization with PECAM-1 (Figure 3B). H8 was weakly expressed in the underlying choroidal vessels during the time course (data not shown). Collagen type IV staining always overlapped with that of H8 but encompassed a larger area at CNV lesion sites (Figure 3C). Staining of whole eye sections, similar to those shown in Figure 2, were used as control to show that localization of H8 staining to the CNV lesion site was not due to mechanical disruption of collagen type IV when peeling away the neuroretina during sample preparation.

**H8 inhibits laser-induced choroidal neovascularization:** Since H8 staining appeared to precede and eventually colocalize with the area of CNV, we wished to evaluate whether in vivo treatment with systemic H8 could reduce CNV lesion size. Indeed, CNV lesion size was markedly reduced following two weeks of i.p. H8 injection as compared to isotype-matched controls (Figure 4A,B). Mice treated with 10 mg/kg of H8 had a moderate but statistically significant decrease in the area of CNV (Figure 4B). Mice treated with 30 mg/kg of H8 had a greater decrease in the size of CNV lesions (Figure 4B).

**DISCUSSION**

**AMD characterized by CNV is a result of pathologic angiogenesis.** As angiogenesis is an invasive process that requires proteolysis of the ECM as well as migration and proliferation of endothelial cells, multiple stimuli may be involved in the development of CNV. Anti-angiogenesis therapies for CNV have focused on targeting vascular endothelial growth factor (VEGF) [27,28], a specific endothelial cell mitogen and permeability factor, and proteolytic enzymes such as MMPs [19-21]. As the pathogenesis of angiogenesis becomes better understood, new targets for CNV are emerging. In the present study, we focused on proteolytic cleavage of the ECM, specifically the proteolytic exposure of a cryptic epitope within collagen type IV that has recently been shown to be required for pathological angiogenesis [24,25].

Recent studies have also indicated that proteolytic enzymes such as MMPs play an important role in angiogenesis [29,30]. In fact, mice deficient for MMP-2 or MMP-9 exhibit reduced CNV [31-33], suggesting that proteolytic remodeling plays an important role in development of CNV. A cryptic collagen type IV epitope can be exposed by proteolytic activity [24]. Indeed, the immunoreactivity of antibodies against these cryptic epitopes, such as H8, greatly increased in the area around the blood vessels of the retina and choroid following MMP-2 treatment. Furthermore, it has been reported that MMP-9 deficient mice exhibit reduced exposure of the cryptic collagen type IV epitopes within the retina during hypoxia-induced retinal neovascularization [25]. Importantly, this retinal neovascularization was inhibited by systemic administration of HUIV26, an alternate antibody that specifically recognizes cryptic collagen type IV epitopes [25]. In agreement with these observations, we show that expression of H8 following laser treatment is preceded by MMP-2 expression and that systemic administration of H8 significantly reduced the size of CNV lesions. Together, these findings support the hypothesis that proteolysis of collagen type IV is one of the critical steps in angiogenic events such as CNV.

We show that during the initial stages of CNV, MMP-2 expression precedes the exposure of the cryptic collagen type IV site recognized by H8. This is in agreement with a previous report that described exposure of cryptic epitopes within the initiation phase of angiogenesis in hypoxia-induced retinal neovascularization [25]. Exposure of cryptic matrix epitopes may represent one of the earliest structural remodeling events that occur before ocular neovascularization, therefore, reagents able to recognize and block access to these epitopes might detect and prevent the ECM damage that leads to clinical neovascularization. Cryptic collagen type IV epitopes are normally hidden within the collagen triple helical structure, but become exposed within the subendothelial basement membrane of angiogenic and tumor associated blood vessels [24]. We show that these sites are also revealed following laser injury CNV. Thus, targeting cryptic collagen type IV epitopes with reagents such as H8 could be a specific therapy for CNV.

Little is known about the mechanisms by which antibodies directed to cryptic collagen type IV epitopes inhibit angiogenesis. Recent studies have shown that MMP-2-mediated cleavage of laminin 5 can expose a cryptic epitope that potentiates breast tumor cell migration [34] and that proteolytic cleavage of fibronectin and osteopontin enhances endothelial cell migration in vitro [35,36]. These findings suggest that proteolytic cleavage of ECM may regulate cell behavior and the cellular interaction with the ECM. Importantly, exposure of a cryptic collagen type IV epitope could enhance retinal endothelial cell migration, and in fact, the HUIV26 antibody from which H8 was derived, did inhibit retinal endothelial cell migration in culture [25]. Our data demonstrate that exposure of cryptic sites was detected in the early stage of CNV development and that H8 antibody administration inhibited the development of CNV as compared to the IgG control. We speculate that H8 may inhibit the development of CNV by blocking endothelial cell migration in the initial stage of angiogenesis.

In conclusion, the current data demonstrate that cryptic collagen type IV epitopes were revealed in laser-induced CNV. Systemic administration of H8 antibody recognizing cryptic collagen type IV epitopes inhibited the development of CNV, suggesting that exposure of a cryptic collagen type IV epitope
is associated with the incidence of CNV. Our findings support the hypothesis that targeting cryptic epitopes within the ECM could lead to new therapies for treatment of CNV.

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REFERENCES

