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Expression of adiponectin in choroidal tissue and inhibition of laser induced choroidal neovascularization by adiponectin

Puran S. Bora[a,*], Sankaranarayanan Kaliappan[a], Valeriy V. Lyzogubov[a], Ruslana G. Tytarenko[a], Sushma Thotakura[b], Tito Viswanathan[b], Nalini S. Bora[a]

[a] *Department of Ophthalmology, Jones Eye Institute, Pat & Willard Walker Eye Research Center, 4301 West Markham, University of Arkansas for Medical Sciences, Little Rock, AR 72205, USA*
[b] *Department of Chemistry, University of Arkansas at Little Rock, 2801 S. University Avenue, Little Rock, AR 72204, USA*

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Abstract The aim of this study was to investigate the role of adiponectin (APN) in a mouse model of laser induced choroidal neovascularization (CNV). We have shown by immunohistochemistry that the expression of APN, adiponectin receptor 1, adiponectin receptor 2 and T cadherin gradually increased from day 1 to day 7 post-laser in laser treated mice compared to controls. Recombinant APN (rAPN) was injected intraperitoneally (i.p., 25 μg/mouse) or intravitreally (2 μg/eye) in lasered mice. Another set of lasered mice received APN peptide via i.p. (75 μg/mouse) or intravitreal (30 μg/eye) route. Control mice received a similar treatment with PBS, control protein or control peptide after laser treatment. We found that in the i.p. and intravitreal injection of rAPN resulted in 78% and 68% inhibition respectively in the size of CNV complex compared to control mice. Similar results were observed when APN peptide was injected intravitreally or i.p. Treatment with rAPN or the peptide resulted in decreased levels of vascular endothelial growth factor. Thus, APN inhibited choroidal angiogenesis and may have therapeutic implications in the treatment of wet age related macular degeneration.

Keywords: Choroid; Neovascularization; Angiogenesis; Adipocyte; Macular degeneration and adiponectin

1. Introduction

Adiponectin (APN) is an adipocyte-specific plasma protein, secreted by adipose cells and mimics many metabolic actions of insulin [1]. APN shares sequence homology with a family of proteins showing a modular design containing a C-terminal complement factor C1q-like globular domain and the C-terminal globular domain of APN is also strikingly similar to that of tumor necrosis factor-alpha (TNF-α) [1,2]. A full cDNA encoding the adiponectin receptor 1 (AdipoR1) and adiponectin receptor 2 (AdipoR2) has been isolated from the mouse as well as from the humans [3]. APN acts via two receptor isoforms, AdipoR1 and AdipoR2 [3]. AdipoR1 and AdipoR2 contain seven transmembrane domains, but are structurally and functionally different from G protein-coupled receptors [4–10].

Plasma APN concentrations were significantly lower in patients with obesity, type II diabetes and coronary artery disease [11]. The decreased plasma APN concentration in diabetes has been reported to be an indicator of macroangiopathy [12,13]. APN may be an important link between insulin resistance, type II diabetes and atherosclerosis and thus could be an important target for future diabetes therapy [14,15]. There are few reports regarding the role of APN in angiogenesis and one report suggested that APN is an anti-angiogenic protein [16,17]. The role of APN in the eye has not been studied. The present study was designed to investigate the role of APN in choroidal angiogenesis by using rodent model of laser-induced choroidal neovascularization (CNV) [18–24]. Age related macular degeneration (AMD) is the leading cause of blindness in the Western world in individuals over the age of 55 years. Our results presented here suggest that APN and APN receptors are present in the eye and the recombinant adiponectin (rAPN) or peptide generated from APN may have therapeutic potential in the treatment of CNV associated with wet AMD as well as in other angiogenesis-dependent diseases like cancer, atherosclerosis and obesity.

2. Methods

2.1. Materials

C57BL/6 mice 4–6 weeks old were purchased from the Jackson Laboratory (Bar Harbor, ME). This study was approved by the Institutional Animal Care and Use Committee (IACUC), University of Arkansas for Medical Sciences (UAMS), Little Rock, AR.

2.2. Immunohistochemistry

The following were used for the lasered mice: goat polyclonal anti-adiponectin antibody (R&D Systems, Minneapolis, MN) in a 1:200 dilution; rabbit anti-adiponectin receptor 1serum (Phoenix Pharmaceuticals, Belmont, CA) – 1:1600; rabbit anti-adiponectin receptor 2 serum (Phoenix Pharmaceuticals) – 1:2000; rabbit anti-T-cadherin (Santa Cruz, CA) – 1:300; secondary rabbit anti-goat IgG (H+L) FITC conjugate (Invitrogen, San Francisco, CA) and Alexa Fluor goat anti-rabbit IgG (H+L) (Invitrogen) – in a 1:400 dilution [18,19]. Semiquantitative scaling of positive signal was performed. The intensity of fluorescence was graded from 0 to 3 (0 – no staining; 1 – faint;

*Corresponding author. Fax: +1 501 686 8316.
E-mail address: pbora@uams.edu (P.S. Bora).

Abbreviations: CNV, choroidal neovascularization; AMD, age related macular degeneration; APN, adiponectin; VEGF, vascular endothelial growth factor; FGF, fibroblast growth factor; rAPN, recombinant adiponectin; AdipoR1, adiponectin receptor 1; AdipoR2, adiponectin receptor 2; Tcad, T cadherin

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2 – moderate; 3 – intense). Please see supplementary material for more information.

2.3. RT-PCR analysis

Ten laser spots were placed in each eye of C57BL/6 mice as described below. Animals from rAPN treated and peptide treated groups ($n = 10$) were sacrificed at days 3 post-laser treatment; RPE-choroidal tissues harvested from the enucleated eyes were pooled separately for each group, and total RNA was prepared using SV Total RNA Isolation kit (Promega) [19]. RT-PCR was conducted using the following primers: **β-actin** (F, 5′-GCC ACC AGT TCG CCA TGG ATG A-3′; R 5′-GTC AGG CAG CTC ATA GCT CTT C-3′); **VEGF** (F, 5′-GCGGGCTGCCTCGCAGTC-3′; R, 5′-TCACCGCCTTGGCTTGTCAC- 3′). Please see supplementary material for more information.

2.4. Western Blot analysis

RPE-choroidal tissues from rAPN treated and peptide treated groups ($n = 10$, at day 3, post-laser treatment) were collected, homogenized and solubilized in ice cold PBS (2.0 ml/eye) containing protease inhibitors, phenylmethylsulfonyl fluoride (1 μg/ml), aprotinin (1 μg/ml), and EDTA (1 mM). The homogenates were centrifuged at $18{,}000 \times g$ at 4 °C for 30 min [19,25]. Please see supplementary material for more information.

2.5. Induction of CNV in mice and APN treatment

Animals (C57BL/6 mice) are divided in to following groups, 1, rAPN treated i.p.; 2, rAPN treated intravitreal; 3, control treated with PBS i.p.; 4, control treated with β-actin i.p.; 5, control treated with PBS intravitreal; 6, control treated with β-actin intravitreal. CNV was induced by laser photocoagulation with the Argon laser (50 μm spot size; 0.05 s duration; 250 mW) as previously described by us [18,19,25]. Three laser spots were placed in each eye close to the optic nerve. In group 1, 5 mice were treated i.p. with 25 μg/mouse of rAPN, 1 day before laser photocoagulation and every day after laser treatment until day 6. In group 2, mice ($n = 5$) were injected 2 μg (single injection) (intravitreal) on day 1. Two controls were used for each group. Controls ($n = 5$) were injected with β-actin or PBS (either i.p. or intravitreal). All the experiments were repeated three times. Please see supplementary material for more information.

2.6. Peptide administration

A region of APN (globular domain) which has no homology with C1q (complement protein) was selected to synthesize the peptide, NH2-KDKAVLFTYDQYQEKNVD – COOH. A control peptide, NH2-FSVGLETRVTVPNVPIRF – COOH was also synthesized from the same region of APN which has no homology with C1q (synthesized at Peptide Biochemical Research Inc., Seattle, USA; dissolved in PBS). In one group, seven i.p. injections of the peptide (75 μg/mouse) were given, one preceding laser treatment and six following the laser treatment. The second group ($n = 5$) was photocoagulated on day 0 followed by a single 30 μg intravitreal injection. Controls for each group were given control peptide or PBS injections. All the experiments were repeated three times. Please see supplementary material for more information.

2.7. Three dimensional (3D) structures

All the molecular modeling calculations were performed using SYBYL program, package version 7.0 on a Silicon Graphics octane system with IRIX 6.5 operating system. The structure of the peptide was built using the 'build protein' tool in SYBYL. Total energies of the various conformations of the polypeptide were calculated, and the least energy conformer was identified. The total energy of the lowest energy conformer for the peptide was calculated to be 43.145 hartrees. Please see supplementary material for more information.

2.8. Measurement of CNV

Seven days after laser treatment, all animals were perfused with 1 ml of PBS containing 50 mg/ml fluorescein-labeled dextran (FITC-dextran; average molecular mass, 2×10^6; Sigma–Aldrich) and sacrificed. The eyes were harvested and fixed in 10% phosphate-buffered formalin, and retinal pigment epithelium (RPE)-choroid-scleral flat mounts were prepared as previously described [18,19]. Please see supplementary material for more information.

2.9. Statistical analysis

Data were subjected to equality of variance F test and ANOVA using Statistica program. Differences were considered statistically significant with $P < 0.05$.

3. Results

3.1. Expression of APN in mouse choroid

Within the normal eye, weak expression of APN was observed in the choroidal tissue. AdipoR1, AdipoR2 and T cadherin (Tcad) staining was also weak in the normal choroidal tissue (Figs. 1 and S1, please see supplementary data). APN staining increased in laser injured areas on day 1 post-laser and peaked on day 7. Similarly, after the laser injury intensity of AdipoR1 and Tcad staining in the area of injury increased gradually from day 1 and peaked on day 5 compared to the controls (Figs. 1 and S1). Intensity of AdipoR2 staining also increased from day 1 to day 5 compared to the controls (Figs. 1 and S1). So, all known APN receptors were increased during the development of CNV.

3.2. Effect of APN on CNV complex size

In intravitreal rAPN injected mice, we observed a 68% decrease in CNV size compared to control mice (Fig. 2A) and a 78 % decrease in the CNV complex size in mice injected intraperitoneally (i.p.) with rAPN compared to controls (Fig. 2B). The values in each group were averaged from 90 laser spots. The confocal images showed inhibition of CNV in intravitreal injected animals (Fig. 3C) compared to controls (Fig. 3A and B). Similarly, i.p. APN injection showed inhibition of CNV (Fig. 3F) compared to controls (Fig. 3D and E).

3.3. Effect of APN peptide on CNV size

A significant inhibition ($P < 0.005$) in the CNV size in APN peptide treated mice compared to controls was observed in animals injected intravitreally (66% inhibition) (Fig. S2A). Similarly, a significant inhibition ($P < 0.005$) in the CNV size in APN peptide treated mice compared to controls was observed in animals injected i.p. (77% inhibition) (Fig. S2B). The values in each group were averaged from 90 laser spots.

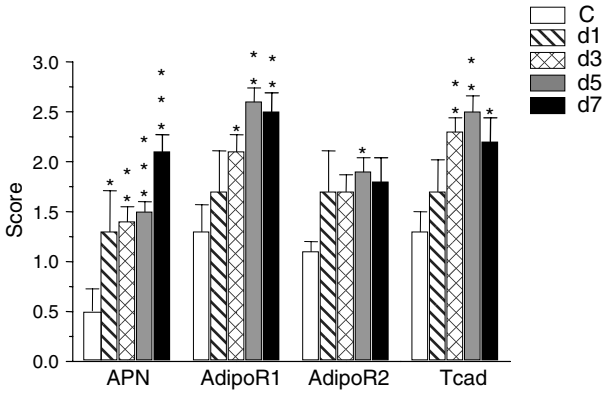

Fig. 1. Semiquantitative scoring of immunohistochemical staining for APN, AdipoR1, AdipoR2 and Tcad during development of CNV (M ± S.E.). In figures: 1d – 1 day; 3d – 3 days, 5d – 5 days and 7d – 7 days after laser injury. Data were subjected to ANOVA/MANOVA analysis. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. All values compared to control. $n = 13$–19 per group.

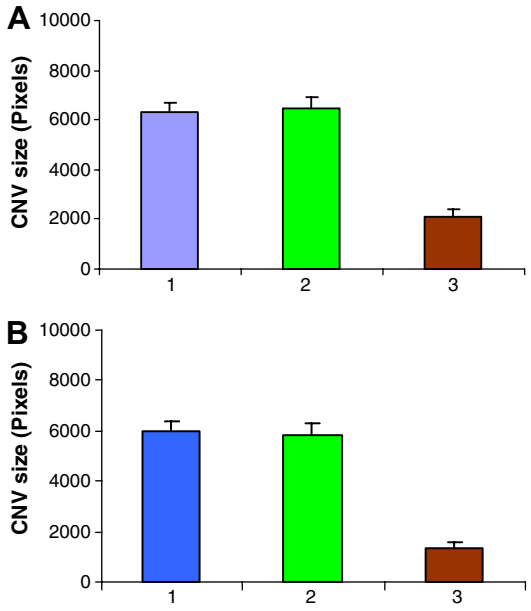

Fig. 2. Measurement of CNV size in APN injected mice. APN was injected intraperitoneally (i.p.) and intravitreally. The control mice were injected with PBS i.p. or intravitreal. CNV size was measured in pixels by using Image Pro-Plus software. There was an 68% decrease ($P < 0.005$) in CNV size in intravitreally injected mice compared to controls (control peptide or PBS injected mice), (A) and 78% decrease in i.p. injected mice, compared to control (PBS injected) mice, (B). The values in each group were averaged from 90 laser spots.

The confocal images shown here represent the images used to calculate the CNV size (Fig. S3A–F, please see supplementary data). The confocal images showed that the neovascular complex was significantly inhibited in the laser spots of C57BL/6 mice treated with APN peptide intravitreally (Fig. S3C) compared to controls (Fig. S3A and B) and i.p. injected mice (Fig. S3F) compared to controls (Fig. S3D and E).

3.4. Expression of growth factors

Animals were sacrificed at day 3 post-laser and RPE-choroid tissues harvested from the enucleated eyes were pooled separately to detect the mRNA and protein levels of β-actin and vascular endothelial growth factor (VEGF). The expression of VEGF mRNA (Fig. 4A) and protein (Fig. 4B) in the choroidal tissue at day 3 post-laser was significantly inhibited in rAPN or APN peptide treated mice compared to control animals.

4. Discussion

APN structurally belongs to the complement protein C1q family [17,26–31]. In the present investigation we have studied the role of APN (30 kDa) in the murine model of laser-induced CNV. To the best of our knowledge this is the first report describing the role of APN in CNV model.

We have shown that very low levels of APN, AdipoR1, AdipoR2 and Tcad are expressed in the normal mice choroidal

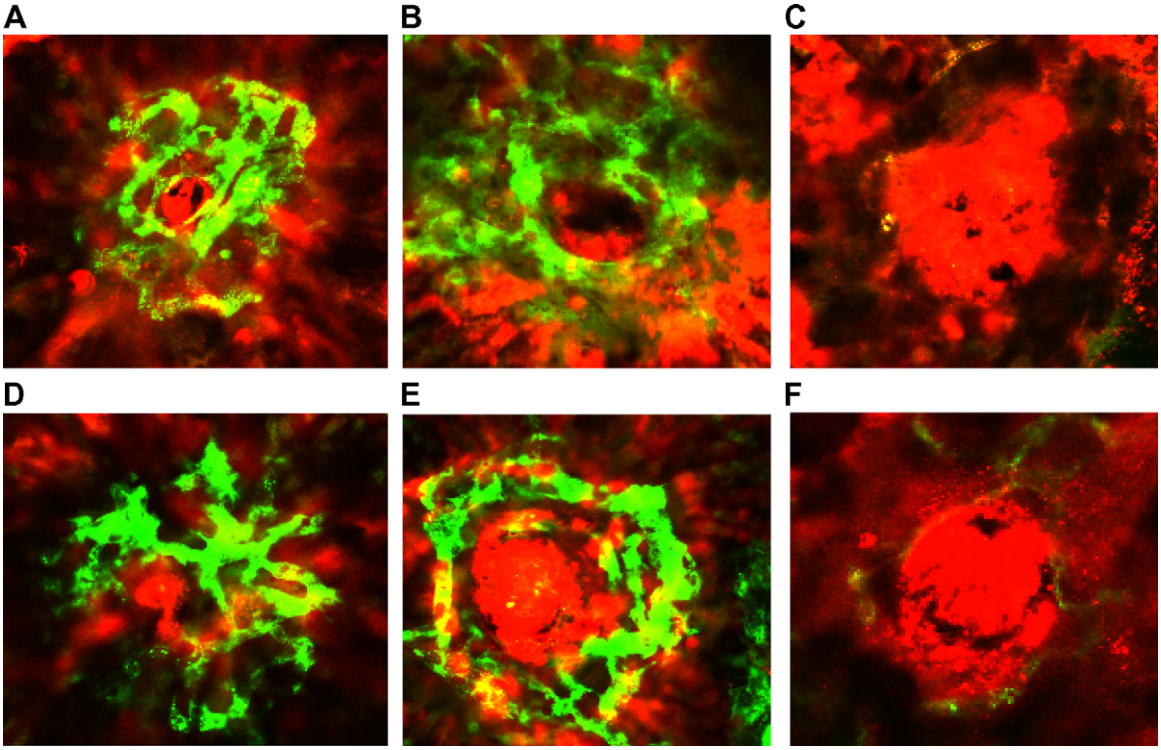

Fig. 3. Confocal micrograph of the neovascular complex in C57BL/6 mice showing the new vessels (green). Laser-induced C57BL/6 mice and control lasered mice were anesthetized and perfused with 1 ml of PBS containing 50 mg/ml fluorescein-labeled dextran. The flat mounts were stained for elastin by using elastin Ab and Cy3-conjugated secondary Ab and were examined by confocal microscope. Elastin stained with red in exposed Bruch's membrane and RPE. Neovascular complexes were significantly inhibited in the laser spots of C57BL/6 mice treated with i.p. injections of APN, (C) compared to controls (A) and (B). Similarly neovascular complex was significantly inhibited in the laser spots of C57BL/6 mice treated with intravitreal injection of rAPN, (F) compared to controls (D) and (E).

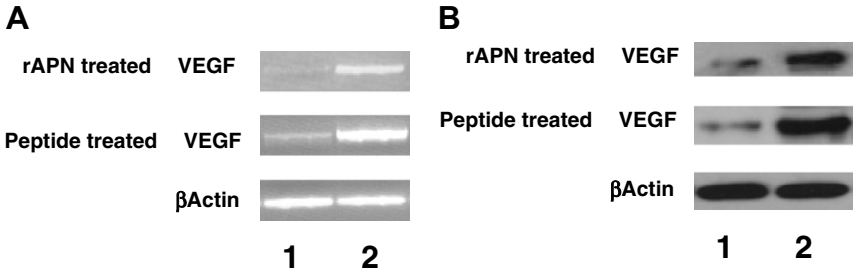

Fig. 4. VEGF mRNA expression in post-laser treated mice. Equal amounts of the total RNA (0.2 μg) were used to detect the mRNA levels of β-actin and VEGF. The expression of VEGF in post lasered, day 3 (APN treated) was significantly decreased lane 1 compared to post-lasered, day 3 (PBS treated), lane 2. A strong band for β-actin indicated equal amounts of RNA in each lane, Fig. 5A. Western Blots were performed using antibodies against VEGF. RPE-choroids were collected from the post-laser groups and were homogenized in solubilizing buffer. The supernatant was used to run on SDS–PAGE. As shown in the Fig. 5B protein levels of VEGF were low in post-laser, day 3 (lane 1) in rAPN or APN peptide treated group compared to control (PBS treated) group, post-laser day 3, lane 2.

tissue and the expression of these proteins slowly increased after laser treatment. Since APN is an anti-angiogenic protein [15–17], low levels of this protein in normal mice may contribute to the development of CNV after laser treatment. APN expression was considered to be limited to adipose tissue until we and other investigators demonstrated that APN is also expressed in the choroidal tissue, iris and ciliary body, cornea and osteoblasts [32–34]. APN expression in cells like hepatocytes, choroid, and skeletal muscle cells can be induced by an inflammatory process [18,19,24,25,35–37] and expected to have increased expression of APN, adipoR1, adipoR2 and Tcad in CNV.

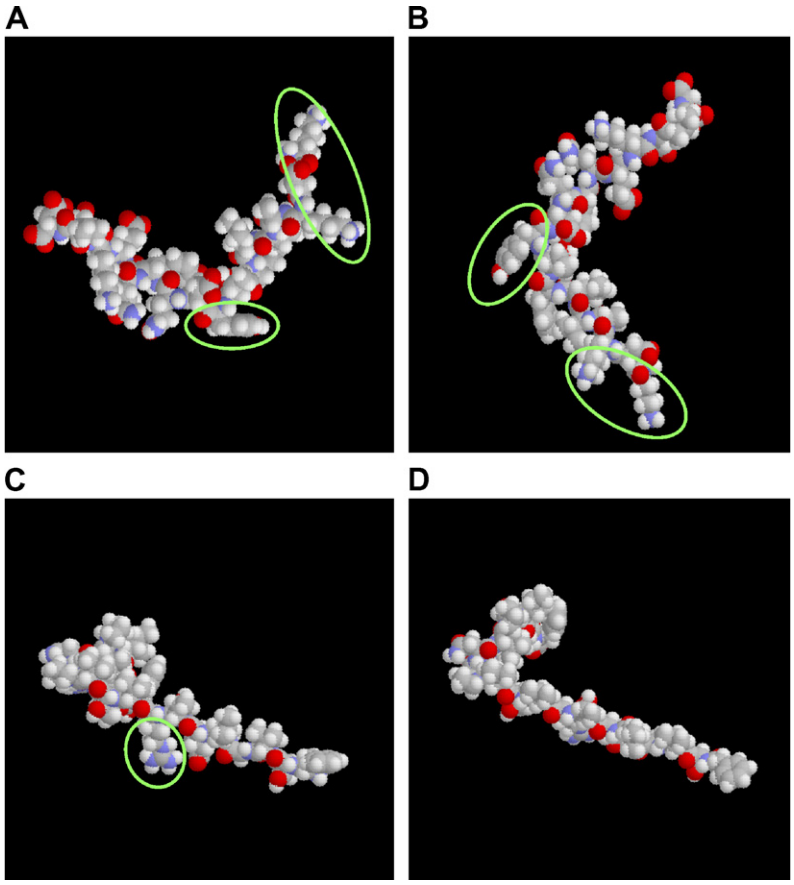

Fig. 5. Three-dimensional (3D) structure of the peptide. Lowest energy conformer of the polypeptide obtained using the SYBYL program. The possible binding site or sites (two amines in close proximity and lysine side chain) are marked by circle (A). Different view of 3D structure of the peptide. Lowest energy conformer of the polypeptide obtained using the SYBYL program. The possible binding site or sites (two amines in close proximity and lysine side chain) are marked by circle (B). Three-dimensional structure of the control peptide. Lowest energy conformer of the polypeptide obtained using the SYBYL program. The possible binding site (valine side chain) is marked by circle (C). Different view of 3D structure of the control peptide. Lowest energy conformer of the polypeptide obtained using the SYBYL program. This 3D view does not show any binding site or sites (D).

The role of APN as the anti-angiogenic agent has been demonstrated by investigators in the mouse tumor model [16,31]. The anti-angiogenic activity of APN encouraged us to determine whether APN could inhibit CNV in the mouse model of wet type AMD or CNV. We have shown that rAPN injection in the laser treated mice inhibited CNV size by 78% in i.p. injected and 68% in intravitreal injected mice. Similarly we have also shown that a peptide generated from the globular region of APN was very effective in inhibiting the development of CNV. This peptide was designed in such a way that they have no sequence homology with C1q. We have shown that C1q does not play any role in the development or inhibition of CNV in this model [25]. This APN peptide was injected i.p. and intravitreally. There was 77% inhibition of CNV in the i.p. treated group and 66% inhibition in the intravitreal group. Thus, our data suggested that the APN peptide was as effective as rAPN in inhibiting the CNV size.

We have determined the 3D structure for both experimental and control peptides. The 3D structure of the peptide showed that in one end of the peptide the amines are in close proximity. Accordingly, this region could be a possible site of interaction of APN and APN receptors and may play an important role in the inhibition of CNV complex. A hook-like side chain of lysine could be another possible interaction site of this peptide and its receptors (Fig. 5A and B). The control peptide also has a hook-like side chain of valine and this may be a possible interaction site of this control peptide to receptors [7,38,39]. However, the interaction of control peptide did not cause inhibition of CNV complex size (Fig. 5C and D). Taken together all the information we believe that the amines in close proximity at one end of the peptide (not present in control peptide) may be contributing in the inhibition of CNV complex size (Fig. 5A and B).

We have shown that rAPN injection in the laser treated mice not only inhibited CNV size but also inhibited the expression of VEGF. VEGF plays a very important role in the development of CNV. When a sufficient amount of APN was present in the choroidal tissue, it inhibited angiogenic factors like VEGF which then inhibited CNV complex size [19,24,25,37].

Thus, in summary we have shown that APN acts as an anti-angiogenic protein in the mouse model of laser-induced CNV. The unique peptide generated from the globular region of APN is also very effective in inhibiting the development of CNV in this model. Since rAPN or APN peptide treatment inhibited VEGF expression, APN or the APN peptide can be used as an alternative therapy for the treatment of AMD or other angiogenesis related diseases.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2007.04.024.

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