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Inhibition of new vessel growth in mouse model of laser-induced choroidal neovascularization by adiponectin peptide II

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Abstract

We have investigated the effect of adiponectin (APN) peptide II on new vessel growth in mouse model of choroidal neovascularization (CNV) or wet type age-related macular degeneration (AMD). Mice were injected intraperitoneally with APN peptide II, control peptide, or PBS on day 1–7 or day 5–14. APN, AdipoR1, PCNA, and VEGF localization was investigated using confocal microscopy, immunohistochemistry, and RT-PCR. APN peptide II decreased the relative area of FITC-dextran perfused vessels by 4-fold, PCNA expression by 3-fold, and the number of PCNA stained HUVEC and MAVEC cells by 38 and 46%, respectively. We concluded that APN peptide II inhibits CNV size on days 7 and 14 by inhibiting the proliferation of endothelial cells in vivo and in vitro. APN peptide II may have therapeutic potential to inhibit CNV or wet AMD.

Keywords

Adiponectin; Angiogenesis; Macular degeneration; Neovascularization; Proliferation

1. Introduction

Adiponectin (APN), an adipokine, structurally belongs to the complement 1q (C1q) family proteins and is composed of an N-terminal collagen-like sequence and C-terminal globular region. The collagen domain modulates APN multimerization and disulfide bonds hold APN oligomers together (Tsao et al., 2003). APN may be enzymatically cleaved to smaller fragments. APN globular domain fragments have metabolic and anti-inflammatory APN activity (Fruebis et al., 2001; Waki et al., 2005). The receptors of APN are adiponectin receptor 1 (AdipoR1), adiponectin receptor 2, and T-cadherin (Yamauchi et al., 2003; Hug et al., 2004). APN receptors mediate adiponectin-induced biological functions (Tsao et al., 2003; Yamauchi et al., 2003; Kadowaki et al., 2006; Ouchi et al., 2000). APN is abundantly present in serum and participates in the regulation of multiple functions: insulin sensitivity and energy homeostasis (Maeda et al., 2002; Vasseur, 2006), inflammation (Wittchen et al., 2007; Kaser et al., 2005; Rovin et al., 2005), cell proliferation (Arita et al., 2002; Motoshima et al., 2004; Ouchi et al., 2004; Shibata et al., 2004), and apoptosis (Kobayashi et al., 2004; Bräkenhielm et al., 2004). APN is produced by adipocytes (Scherer et al., 1995) with other

cells (Wittchen et al., 2007; Kaser et al., 2005; Rovin et al., 2005; Chen et al., 2006) and secreted into the bloodstream.

One of the pathological complications of age-related macular degeneration (AMD) is choroidal angiogenesis or neovascularization (CNV) (Ferris et al., 1984; Zarbin, 2004). AMD has two phenotypes: (1) dry type, characterized by the presence of drusen; and (2) wet type, characterized by new vessel formation (Bora et al., 2005; Campochiaro, 2000) from choroid or CNV. Wet type AMD can cause legal blindness and the disease is irreversible in nature.

We previously reported that adipoR1 and APN expression increased in CNV mouse laser-induced model (Bora et al., 2007), and proposed that APN may contribute to CNV development. We also demonstrated that APN and APN peptide from the APN globular region inhibited CNV. However, target cells for APN and APN peptide action and mechanisms of the APN anti-angiogenic action required further investigations. The objective of the present study was to investigate the effects of APN peptide II on new vessel growth in the CNV mouse model. We show that APN and adipoR1 are co-localized in endothelial cells of newly formed vessels in the mouse choroid. Systemic administration of APN peptide II inhibited proliferation of endothelial cells and decreased CNV size. Proliferation also decreased when human umbilical vein endothelial cell (HUVEC) and mouse aortic vascular endothelial cell (MAVEC) were treated with APN peptide II in vitro.

2. Materials and methods

2.1. Experimental animals

Male C57BL/6 mice – 6 weeks old (from the Jackson Laboratory, Bar Harbor, ME) were used. 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. Three-dimensional (3D) structure of APN peptide

A region of APN (globular domain) was selected to synthesize the APN peptide II (NH₂-LQVYGDGDHN-GLYADNVN-COOH) and the control peptide (NH₂-FSVGLETRVTPNVPPPIRF-COOH) (Peptide Biochemical Research Inc., Seattle, WA). Molecular modeling calculation was performed using SYBYL program, package version 7.0 on a Silicon Graphics octane system with IRIX 6.5 operating system. The peptide's structure was built using the 'build protein' tool in SYBYL. Total energies of the various poly-peptide conformations 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.

2.3. Induction of CNV in mice and APN peptide injection

Animals were divided into five groups of 12 animals. CNV was induced by Argon laser photocoagulation (spot size – 50 µm; duration – 0.05 s; power – 260 mW) as previously described by us (Bora et al., 2003, 2005). Three laser spots were placed in each eye close to the optic nerve. Animals were injected daily intraperitoneally: group 1 received 200 µg of APN peptide II prepared on 0.1 ml of sterile PBS; group 2 received 200 µg of control peptide dissolved in PBS; and group 3 got 0.1 ml sterile PBS. Animals were sacrificed at day 7 or 14 after laser injury.

2.4. Immunohistochemistry, morphometry and in vitro experiments

Mouse eyes at day 7 after laser ($n = 7$) were fixed in 10% formalin solution (Sigma–Aldrich, St. Louis, MO) and embedded in paraffin. Four-micron serial sections were cut. After deparaffinization in xylene and hydration, immunohistochemical (IHC) reactions were

performed. Goat polyclonal anti-adiponectin antibody, diluted 1:400, and goat polyclonal anti-vascular endothelial growth factor (VEGF) antibody, diluted 1:200, (R&D Systems, Minneapolis, MN); rabbit anti-adiponectin receptor 1 serum, diluted 1:400, (Phoenix Pharmaceuticals, Belmont, CA); goat polyclonal anti-proliferating-cell nuclear antigen (PCNA) antibody diluted 1:2000 (Biovision, Mountain View, CA) were used. Isolectin IB4-AF594; secondary rabbit anti-goat IgG (H + L)-FITC, rabbit anti-goat IgG (H + L)-AF594, goat anti-rabbit IgG (H + L)-AF488 (Invitrogen, South San Francisco, CA), all diluted 1:400, were used. After IHC, labeling sections were mounted in ProLong antifade reagent with DAPI (Invitrogen, South San Francisco, CA). Observation and capturing of sections were made using an Olympus microscope equipped with QImaging Go-5 camera (Surrey, BC, Canada). Three sections from each laser injured area were captured and a total of 21 laser spots sections per group were investigated. Number of PCNA positive nuclei and total number of nuclei in laser injured area were counted; then percent of PCNA positive nuclei was calculated. For in vitro experiments, we followed the method of Nishiyama et al. (2007) to grow the HUVEC and MAVEC cells in a Petri dish. These cells were treated with 200 µg of APN peptide II when they were half confluent and 42 h later stained for PCNA (green) by adding PCNA antibody. The cells were fixed before adding the PCNA antibody. As mentioned above the PCNA (green) stained cells were analyzed and counted under the microscope.

2.5. Measurement of CNV

Animals from APN peptide II and control groups were perfused on days 7 and 14 with PBS containing 50 mg/ml fluorescein-labeled dextran (Sigma-Aldrich, St. Louis, MO) and sacrificed. The eyes were harvested, fixed in formalin, and retinal pigment epithelium (RPE)-choroid-scleral flat mounts were prepared as previously described (Bora et al., 2003; Kimura et al., 1995). Images were captured using confocal microscopy. The relative area of fluorescein isothiocyanate (FITC)-dextran perfused vessels in laser injured zones was measured with ImagePro Plus software (Media Cybernetics, Inc. Bethesda, MD).

2.6. RT-PCR analysis

Three laser spots were placed in each eye of mice as described above. Five animals from each group were sacrificed at day 3 and RPE-choroid-sclera tissues were harvested from the enucleated eyes. Total RNA was extracted from the tissue with RNeasy Mini Kit (QIAGEN, Valencia, CA). Equal amounts of the total RNA (0.1 µg) were used to detect the mRNA levels of β -actin and VEGF using the reagents from Applied Biosystems (Foster City, CA). Oligonucleotide primers were obtained from Integrated DNA Technologies (San Diego, CA). The negative controls consisted of omission of RNA or reverse transcriptase from the reaction mixture. PCR products were analyzed on a 1% agarose gel and were examined by using Quantity One (Bio-Rad, Hercules, CA). RT-PCR was conducted using the following primers:

β -actin (746 bp)

forward: 5'-GCC ACC AGT TCG CCA TGG ATG A-3',

reverse: 5'-GTC AGG CAG CTC ATA GCT CTT C-3';

VEGF (716 and 512 bp)

forward: 5'-GCG GGC TGC CTC GCA GTC-3',

reverse: 5'-TCA CCG CCT TGG CTT GTC-3'.

2.7. Statistical analysis

Comparison between groups was performed with Statistica program using Student *t*-test analysis. Data presented as mean $\pm$ SE. Significance was defined at $p < 0.05$.

3. Results

3.1. APN and AdipoR1 localization in endothelial cells of newly formed vessels

Laser-induced CNV is an animal model of wet AMD (Bora et al., 2005; Kimura et al., 1995). Our investigation of the vessel growth and inflammation after choroid injury may help to understand the mechanisms of vessel formations in human pathology. We hypothesized that APN may inhibit CNV in mice. We stained serial sections of mouse choroids at day 7 after laser photocoagulation using IHC method with antibodies against APN and AdipoR1. Double staining has shown that both APN and adipoR1 were present in endothelium of newly formed vessels of the mouse choroid and co-localized in apical surface area of endothelial cells (Fig. 1). Thus, CNV formation may be dependent on APN because endothelial cells express adipoR1 and APN and may be sensitive to APN action.

3.2. APN peptide II inhibition of CNV size

Previously, we reported the inhibitory effect of recombinant APN and APN peptide I corresponding to 180–198 amino acids sequence of mouse APN globular domain on CNV (Bora et al., 2007). To confirm APN's anti-angiogenic effect, we tested the effect of another APN peptide II corresponding to 216–233 amino acids sequence of mouse APN globular domain. We injected mice with APN peptide II, control peptide prepared in PBS or PBS i.p. daily until day 7 and from day 5 to day 14, and then animals were perfused with FITC-dextran and sacrificed. We demonstrated by confocal microscopy that i.p. injection of APN peptide II significantly decreased CNV size compared to PBS and control peptide treated groups (Figs. 2 and 3).

The 3D structure of the APN peptide II showed presence of a hook-like side chain (Fig. 4B). This region could be a possible region of interaction with APN receptors. The 3D structure of control peptide is also shown (Fig. 4A). We have shown that control peptides did not inhibit CNV.

3.3. APN peptide II inhibits expression of growth factor VEGF and proliferative marker PCNA

After confirmation of the anti-angiogenic effect of APN peptide II, we hypothesized that this peptide may inhibit VEGF expression and proliferation of endothelial cells. We studied eye samples from experimental groups by using RT-PCR analysis and found decrease of VEGF expression on day 3 after laser treatment (Fig. 5A, B). Immunohistochemical investigation of choroidal sections also showed decrease of VEGF (purple color) in APN peptide II treated group (Fig. 5C) compared to controls (Fig. 5D). We stained serial sections of the eyes from experimental groups with endothelial marker isolectin IB4 and proliferative marker PCNA. We found that isolectin IB4 positive cells were located in choroid and within subretinal space after laser injury. The distribution of isolectin IB4 positive cells in APN peptide II and control peptide treated animals was the same (Fig. 6A and B). In contrast, we found that PCNA positive proliferating cells were less numerous in subretinal tissue of APN peptide II treated group (Fig. 6B) compared to the control (Fig. 6A). The PCNA positive proliferating cells were only 4% in subretinal tissue of APN peptide II-treated group compared to 12% in PBS-(control) treated group (Fig. 6C). In vitro studies showed that 200 μ g of APN peptide II treated HUVEC and MAVEC cells had 38% and 46% less PCNA positive cells, respectively compared to untreated cells.

4. Discussion

The role of APN in the eye has not been studied; so present study was designed to investigate the role of APN in choroidal angiogenesis using a mouse model of laser-induced CNV (Bora et al., 2005, 2007; Campochiaro, 2000). AMD is the leading cause of blindness in the Western world among individuals over age 50. Although the non-exudative (dry) form of AMD is more prevalent, catastrophic vision loss is more frequently associated with the exudative (wet) form, specifically from the complication of neovascularization of the choroid (Ferris et al., 1984; Zarbin, 2004; Bora et al., 2005; Campochiaro, 2000). We have previously shown that APN and APN receptors are present in the eye and the recombinant APN or peptide generated from APN may have therapeutic potential in CNV treatment associated with wet AMD, as well as, in other angiogenesis-dependent diseases like cancer, atherosclerosis and obesity.

We synthesized five peptides (each of 18 amino acids) from globular region of adiponectin, which has no homology with the complement component 1q (C1q), and were named Peptide I, II, III, IV, and V, with Peptide I located near the N-terminal and Peptide V located near the C-terminal. Before studying selective peptides in detail, we injected the five peptides separately into mice, which were then laser photo-coagulated to induce CNV. Only Peptides I and II inhibited CNV. Peptides III, IV, and V had no effect on CNV. APN peptide II and APN peptide III (control peptide) were used for detailed CNV studies. We remain unsure why only APN peptide I (Bora et al., 2007) and APN peptide II have inhibitory effect on CNV, and therefore structural differences in these peptides are under investigation.

Investigators have shown that APN has stimulatory (Ouchi et al., 2004; Shibata et al., 2004) or inhibitory (Motoshima et al., 2004) effect on proliferation of endothelial cells and angiogenesis. We have shown that APN peptide II inhibited proliferation of endothelial cells by inhibiting VEGF expression at final stages of differentiation and prevented growth of new vessels. APN peptide II inhibited vessel growth in the CNV mouse model or wet type AMD on days 7 and 14. Inhibition of CNV size on day 14 was particularly significant because patients visit their eye physician when AMD has already developed. In this mouse model, CNV is fully developed on day 7. We stained serial sections of the eyes from experimental groups with endothelial marker isolectin IB4 and proliferative marker PCNA. We found that isolectin IB4 positive cells were located in choroid and within subretinal space after laser injury. Since the distribution of isolectin IB4 positive cells in APN peptide II treated and control animals was the same, this may mean that APN peptide II did not affect the subretinal cellular mass formation. In contrast, we found that PCNA positive proliferating cells were less numerous in subretinal tissue of APN peptide II treated group compared to the controls. The PCNA positive proliferating cells were only 4% in subretinal tissue of APN peptide II treated group compared to 12% in PBS-(control) treated group. This indicates that APN peptide II decreased proliferation by 3-fold thus inhibiting new vessel formation in this model. Similarly, the number of PCNA positive HUVEC and MAVEC in APN peptide II treated cells were decreased 38 and 46% respectively compared to untreated cells indicating that APN peptide II inhibited proliferation of these cells.

We demonstrated that APN peptide II is a potent CNV inhibitor in the mouse laser-induced CNV model. The unique peptide generated from the APN globular region is very effective in inhibiting CNV development in this model. Since APN peptide II treatment inhibited VEGF expression, APN peptide II can be used as an alternative therapy for AMD treatment. Furthermore, the use of small molecules, such as APN peptide II, will be cost effective in AMD treatment. Our future studies in this area will focus on the use of APN peptides in CNV inhibition and other angiogenesis-related diseases.

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Abbreviations

CNV	choroidal neovascularization
AMD	age-related macular degeneration
VEGF	vascular endothelial growth factor
RPE	retinal pigment epithelium
FITC	fluorescein isothiocyanate
APN	Adiponectin
PCNA	proliferating-cell nuclear antigen
IHC	immunohistochemical
HUVEC	human umbilical vein endothelial cell
MAVEC	mouse aortic vascular endothelial cell

References

- Arita Y, Kihara S, Ouchi N, Maeda K, Kuriyama H, Okamoto Y, et al. Adipocyte-derived plasma protein adiponectin acts as a platelet-derived growth factor-BB-binding protein and regulates growth factor-induced common postreceptor signal in vascular smooth muscle cell. *Circulation*. 2002; 105:2893–8. [PubMed: 12070119]
- Bora PS, Hu Z, Tezel TH, Sohn JH, Kang SG, Cruz JM, et al. Immunotherapy for choroidal neovascularization in a laser-induced mouse model simulating exudative (wet) macular degeneration. *Proc Natl Acad Sci USA*. 2003; 100:2679–84. [PubMed: 12589025]
- Bora PS, Sohn JH, Cruz JM, Jha P, Nishihori H, Wang Y, et al. Role of complement and complement membrane attack complex in laser-induced choroidal neovascularization. *J Immunol*. 2005; 174:491–7. [PubMed: 15611275]
- Bora PS, Kaliappan S, Lyzogubov VV, Tytarenko RG, Thotakura S, Viswanathan T, et al. Expression of adiponectin in choroidal tissue and inhibition of laser-induced choroidal neovascularization by adiponectin. *FEBS Lett*. 2007; 581:1977–82. [PubMed: 17466298]
- Bråkenhielm E, Veitonmäki N, Cao R, Kihara S, Matsuzawa Y, Zhivotovsky B, et al. Adiponectin-induced antiangiogenesis and antitumor activity involve caspase-mediated endothelial cell apoptosis. *Proc Natl Acad Sci USA*. 2004; 101:2476–81. [PubMed: 14983034]
- Campochiaro PA. Retinal and choroidal neovascularization. *J Cell Physiol*. 2000; 184:301–10. [PubMed: 10911360]
- Chen J, Tan B, Karteris E, Zervou S, Digby J, Hillhouse EW, et al. Secretion of adiponectin by human placenta: differential modulation of adiponectin and its receptors by cytokines. *Diabetologia*. 2006; 49:1292–302. [PubMed: 16570162]
- Ferris FL III, Fine SL, Hyman L. Age-related macular degeneration and blindness due to neovascular maculopathy. *Arch Ophthalmol*. 1984; 102:1640–2. [PubMed: 6208888]
- Fruebis J, Tsao TS, Javorschi S, Ebbets-Reed D, Erickson MR, Yen FT, et al. Proteolytic cleavage product of 30-kDa adipocyte complement-related protein increases fatty acid oxidation in muscle and causes weight loss in mice. *Proc Natl Acad Sci USA*. 2001; 98:2005–10. [PubMed: 11172066]
- Hug C, Wang J, Ahmad NS, Bogan JS, Tsao TS, Lodish HF. T-cadherin is a receptor for hexameric and high-molecular-weight forms of Acrp30/adiponectin. *Proc Natl Acad Sci USA*. 2004; 101:10308–13. [PubMed: 15210937]

- Kadowaki T, Yamauchi T, Kubota N, Hara K, Ueki K, Tobe K. Adiponectin and adiponectin receptors in insulin resistance, diabetes, and the metabolic syndrome. *J Clin Invest.* 2006; 116:1784–92. [PubMed: 16823476]
- Kaser S, Moschen A, Cayon A, Kaser A, Crespo J, Pons-Romero F, et al. Adiponectin and its receptors in non-alcoholic steatohepatitis. *Gut.* 2005; 54:117–21. [PubMed: 15591515]
- Kimura H, Sakamoto T, Hinton DR, Spee C, Ogura Y, Tabata Y, et al. A new model of subretinal neovascularization in the rabbit. *Invest Ophthalmol Vis Sci.* 1995; 36:2110–9. [PubMed: 7657549]
- Kobayashi H, Ouchi N, Kihara S, Walsh K, Kumada M, Abe Y, et al. Selective suppression of endothelial cell apoptosis by the high molecular weight form of adiponectin. *Circ Res.* 2004; 94:e27–31. [PubMed: 14752031]
- Maeda N, Shimomura I, Kishida K, Nishizawa H, Matsuda M, Nagaretani H, et al. Diet-induced insulin resistance in mice lacking adiponectin/ACRP30. *Nat Med.* 2002; 8:731–7. [PubMed: 12068289]
- Motoshima H, Wu X, Mahadev K, Goldstein BJ. Adiponectin suppresses proliferation and superoxide generation and enhances eNOS activity in endothelial cells treated with oxidized LDL. *Biochem Biophys Res Commun.* 2004; 315:264–71. [PubMed: 14766203]
- Nishiyama T, Mishima K, Ide F, Yamada K, Obara K, Sato A, et al. Functional analysis of an established mouse vascular endothelial cell line. *J Vasc Res.* 2007; 44:138–48. [PubMed: 17215585]
- Ouchi N, Kihara S, Arita Y, Okamoto Y, Maeda K, Kuriyama H, et al. Adiponectin, an adipocyte-derived plasma protein, inhibits endothelial NF-kappaB signaling through a cAMP-dependent pathway. *Circulation.* 2000; 102:1296–301. [PubMed: 10982546]
- Ouchi N, Kobayashi H, Kihara S, Kumada M, Sato K, Inoue T, et al. Adiponectin stimulates angiogenesis by promoting cross-talk between AMP-activated protein kinase and Akt signaling in endothelial cells. *J Biol Chem.* 2004; 279:1304–9. [PubMed: 14557259]
- Rovin BH, Song H, Hebert LA, Nadasdy T, Nadasdy G, Birmingham DJ, et al. Plasma, urine, and renal expression of adiponectin in human systemic lupus erythematosus. *Kidney Int.* 2005; 68:1825–33. [PubMed: 16164660]
- Scherer PE, Williams S, Fogliano M, Baldini G, Lodish HF. A novel serum protein similar to C1q, produced exclusively in adipocytes. *J Biol Chem.* 1995; 270:26,746–9.
- Shibata R, Ouchi N, Kihara S, Sato K, Funahashi T, Walsh K. Adiponectin stimulates angiogenesis in response to tissue ischemia through stimulation of amp-activated protein kinase signaling. *J Biol Chem.* 2004; 279:28,670–4.
- Tsao TS, Tomas E, Murrey HE, Hug C, Lee DH, Ruderman NB, et al. Role of disulfide bonds in Acrp30/adiponectin structure and signaling specificity. Different oligomers activate different signal transduction pathways. *J Biol Chem.* 2003; 278:50810–7. [PubMed: 14522956]
- Vasseur F. Adiponectin and its receptors: partners contributing to the “vicious circle” leading to the metabolic syndrome? *Pharmacol Res.* 2006; 53:478–81. [PubMed: 16678433]
- Waki H, Yamauchi T, Kamon J, Kita S, Ito Y, Hada Y, et al. Generation of globular fragment of adiponectin by leukocyte elastase secreted by monocytic cell line THP-1. *Endocrinology.* 2005; 146:790–6. [PubMed: 15528304]
- Wittchen F, Suckau L, Witt H, Skurk C, Lassner D, Fechner H, et al. Genomic expression profiling of human inflammatory cardiomyopathy (DCMi) suggests novel therapeutic targets. *J Mol Med.* 2007; 85:257–71. [PubMed: 17106732]
- Yamauchi T, Kamon J, Ito Y, Tsuchida A, Yokomizo T, Kita S, et al. Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. *Nature.* 2003; 423:762–9. [PubMed: 12802337]
- Zarbin MA. Current concepts in the pathogenesis of age-related macular degeneration. *Arch Ophthalmol.* 2004; 122:598–614. [PubMed: 15078679]

Fig. 1. Immunohistochemical co-localization of APN (A, red) and AdipoR1 (B, green) in a choroid vessel (v). Sites of APN and adipoR1 co-localization (C, yellow) were close to the apical surface of endothelial cells (arrow). Nuclei were stained with DAPI (blue color).

Fig. 2.

Confocal micrographs of mouse RPE-choroid-sclera flat mounts. Treatment with APN peptide II or control solutions was performed from day 1 to day 7 after laser. CNV (green color) size in PBS (A) and control peptide-treated mice (B) was larger compared to APN peptide II-treated animals (C). APN peptide II significantly decreased relative area of CNV (D). Number of spots in each group was 20. * $p < 0.05$ compared to PBS-treated control; # $p < 0.05$ compared to control peptide treated.

Fig. 3.

Confocal micrographs of mouse RPE-choroid-sclera flat mounts. Treatment with APN peptide II or control solution was performed from day 5 to day 14 after laser. CNV (green color) size in control peptide-treated mice (A) treated mice was larger compared to APN peptide II-treated animals (B). APN peptide II significantly decreased relative area of CNV (C). Number of spots in each group was 20. * $p < 0.05$ compared to control peptide-treated.

Fig. 4. Three-dimensional structures of control peptide (A) and APN peptide II (B). Lowest energy conformer of the polypeptides obtained using the SYBYL program. The possible binding site of APN peptide II to receptor is marked in circle.

Fig. 5. Expression of VEGF in mouse choroid. Total RNA was extracted from the RPE-choroid tissue. PCR products were analyzed on a 1% agarose gel (A). APN peptide II decreased VEGF RNA expression on day 3 after laser. Line 1, control; line 2, APN peptide II (B). Formalin fixed, paraffin embedded section were IHC stained for VEGF. Expression of VEGF (purple color) was significantly lower in choroid of APN peptide II-treated animals (C) compared to control (D).

Fig. 6.

Quantitative immunohistochemical analysis of APN peptide II on PCNA expression in the area of laser injury. Isolectin IB4 positive cells (red color) located in choroid (Ch) and subretinal tissue (SRT) of both groups. PCNA positive green stained nuclei were more numerous in CNV area of control peptide (A), compared to APN peptide II-treated animals (B). APN peptide II significantly decreased (4%) PCNA positive nuclei in the area of laser injury on day 7 compared to control peptide (12%) (C). The numbers of spots investigated per group were 21. $*p < 0.05$.