Inhibition of Choroidal Neovascularization by a Peptide Inhibitor of the Urokinase Plasminogen Activator and Receptor System in a Mouse Model

Arup Das, MD, PhD; Nathan Boyd, BS; Terence R. Jones, PhD; Nicholas Talarico, BS; Paul G. McGuire, PhD

Objectives: To determine the role played by the urokinase plasminogen activator (uPA) and urokinase plasminogen activator receptor (uPAR) system in choroidal neovascularization (CNV) and whether inhibition of this system can suppress the extent of CNV in an animal model.

Methods: Choroidal neovascularization was induced in mice by laser photocoagulation using the slitlamp delivery system. Reverse transcriptase–polymerase chain reaction and immunocytochemical analysis were performed on the retina choroids of these animals to examine the expression of uPAR. For 2 weeks following laser treatment, animals were injected intraperitoneally with a novel peptide inhibitor of the uPA-uPAR system (100 mg/kg twice a day every day, every other day, and once a week). Control laser-treated animals receive an intraperitoneal injection of phosphate-buffered saline every day. Following treatment, animals were perfused with fluorescein-labeled dextran, eyes were removed, and the areas of new vessels were examined in the retina-choroid whole mounts by fluorescence microscopy and quantitated using image analysis software.

Results: In this study, uPAR was found to be upregulated in the choroidal tissues of mice with laser-induced CNV. The uPAR was localized to the endothelial cells of the fibrovascular tissue within the CNV complex. Systemic administration of the peptide inhibitor of the uPA-uPAR system resulted in a significant reduction of CNV (up to 94%). The response was found to be frequency-of-dose dependent. No toxic effects or tissue destruction was noted following the peptide treatment.

Conclusions: Our results strongly suggest that upregulation of the uPA-uPAR system is an important step during CNV, and significant inhibition of CNV was seen when cell surface–associated uPA-uPAR activity was prevented with the peptide inhibitor.

Clinical Relevance: Inhibition of the protease system (uPA-uPAR) may prove to be a potential novel antiangiogenic therapy for CNV as seen in age-related macular degeneration.


©2004 American Medical Association. All rights reserved.

C   OROIDAL NEOVASCULARIZATION (CNV) due to age-related macular degeneration (ARMD) is one of the most frequent causes of vision loss in people older than 65 years. The exudative form of ARMD associated with all the elements of atrophic or nonexudative forms of ARMD, as well as neovascularization, results in sudden, severe central vision loss. Although the series of events that lead to the development of CNV is not totally clear, new vessels derived from the choroid penetrate through the Bruch membrane, become leaky, and cause serious retinal detachment and hemorrhage in the macula.

The current treatment options for CNV are conventional laser treatment and photodynamic treatment, both of which have limitations. The former results in irreversible tissue damage with the resultant central scotoma and vision loss, whereas the latter causes short-term closure of new vessels as they open up again in a few months, and the treatment has to be repeated frequently. This fact has led to the need for a better understanding of the mechanisms of CNV formation and a search for new therapeutic methods to treat it.

A model of CNV has been developed for the mouse that uses laser photocoagulation as the primary stimulus. Disruption of the Bruch membrane following laser treatment results in the formation of new vessels beneath the retina derived from the choroid. This has proved to be a useful model for the identification of the mechanisms involved in subretinal neovascularization and the development and testing of new therapeutic interventions for this medical problem.
The angiogenic process consists of several phases, including up-regulation of angiogenic factors followed by increased expression of integrins and extracellular proteinases. The proteinases facilitate endothelial cell migration and new tube formation following the breakdown of the capillary basement membrane. One proteinase that has been shown to play an important role in angiogenesis is the serine proteinase urokinase plasminogen activator (uPA). The uPA localizes to the surface of endothelial cells by binding to the urokinase plasminogen activator receptor (uPAR). This interaction of uPA and uPAR facilitates cell migration through localized proteolytic and nonproteolytic regulation of cell-substrate adhesion. The uPA-plasmin system may also interface with the matrix metalloproteinase system to perform large-scale proteolytic events that may change the regulatory information contained within the extracellular matrix.

Studies have previously reported the increased expression of uPA along with the matrix metalloproteinases 2 and 9 in mice with retinal neovascularization and in human neovascular membranes obtained from patients with proliferative diabetic retinopathy. Furthermore, experiments from this laboratory have demonstrated the up-regulation of uPAR in a murine model of retinal neovascularization and the inhibition of neovascularization in this model using a novel peptide inhibitor of the uPA-uPAR system, A6. The A6 peptide is derived from the non–receptor-binding region of urokinase and has been shown to inhibit the interaction of uPA with uPAR and inhibit tumor cell invasion in vitro. This peptide has antiangiogenic and antitumor activity in animal models of breast cancer and glioblastoma. Recently, transgenic mice that lacked uPA, tissue-type plasminogen activator, and plasminogen genes have been shown to be resistant to the development of experimental CNV. In the current study, we present data on the expression of uPAR in a mouse model of CNV and the potential of the A6 peptide to serve as an effective and novel antiangiogenic therapy for CNV.

METHODS

ANIMAL MODEL OF CNV

All experiments were conducted in accordance with the Association for Research in Vision and Ophthalmic Statement for the Use of Animals in Ophthalmic and Vision Research. Specific pathogen-free C57BL/6j mice were bred at the University of New Mexico Animal Research Facility. Six- to 8-week-old mice were anesthetized with ketamine hydrochloride (100 mg/kg), and pupils were dilated with 1% tropicamide eyedrops. Aragon green laser photocoagulation was performed with the slit-lamp delivery system using a coverslip as a contact lens as previously described. Three laser burns were placed in each eye at the 9-, 12-, and 3-o’clock positions approximately 2 disc diameters from the optic nerve. The power settings were as follows: 100-μm spot size, 0.1-second duration, and 150-mW intensity. Production of a vaporization bubble during laser treatment was considered as the end point and indicated the rupture of the Bruch membrane. Only those eyes with burns that resulted in formation of the vaporization bubble at all 3 locations were included in this study. After 14 days, animals were killed and eyes collected for analysis.

HISTOLOGIC ANALYSIS

Fourteen days after laser treatment, mice (n=5) were killed, and the area of new vessels at each laser site was measured in retinal pigment epithelium (RPE)–choroidal flat mounts. Mice were anesthetized and perfused with 1 mL of phosphate-buffered saline (PBS) containing 30 mg/mL of fluorescein-labeled dextran (average molecular weight of 2 million; Sigma-Aldrich Co, St Louis, Mo). Eyes were removed and fixed in 10% formaldehyde for 2 hours. After removal of the cornea and lens, radial cuts were made from the edge to the equator, and whole mounts of the RPE-choroid complex were prepared in aqueous mounting medium (Aquamount; BDH Chemicals Ltd, Poole, England) with the sclera facing down. Whole mounts were examined by fluorescence microscopy (Axiovert 35; Carl Zeiss, Inc, Thornwood, NY), and images of the burn areas were collected and analyzed. MetaMorph image analysis software (Universal Imaging Corp, Downingtown, Pa) was used to delineate and measure the area of new vessel-associated fluorescence within the burn area. The areas occupied by new vessels were analyzed by repeated-measure analysis of variance using the SAS statistical analysis software (Proc Mixed procedure; SAS Institute, Cary, NC). Post hoc comparisons between treatments were analyzed using least squares means.

A6 TREATMENT

Some mice were treated during the 14-day period with the inhibitor drug A6 peptide (Angstrom Pharmaceuticals, San Diego, Calif). Intraportal injections were given according to the following regimen: (1) 100 mg/kg twice a day once per week (n=5 animals, 20 laser spots analyzed), (2) 100 mg/kg twice a day every third day (n=5 animals, 17 laser spots analyzed), and (3) 100 mg/kg twice a day every day (n=5 animals, 17 laser spots analyzed). Animals that received PBS twice a day every day served as controls in this experiment (n=5 animals, 19 laser spots analyzed). After 14 days of drug treatment, animals were killed, and the extent of CNV was determined.

IMMUNOHISTOCHEMICAL ANALYSIS

For the localization of uPAR, unfixed sections of eyes that contained laser burns were blocked with 10% normal goat serum for 30 minutes followed by incubation with the primary rabbit anti-mouse uPAR antibody (R&D Systems, Minneapolis, Minn) for 1 hour at room temperature. Sections were washed and incubated with an alkaline phosphatase–labeled goat anti-rabbit secondary antibody for 1 hour at room temperature. Positive staining was detected using the Vectastain Vector Red reagent (Vector Laboratories, Burlingame, Calif). Control sections included those without any primary antibody treatment.

REVERSE TRANSCRIPTASE–POLYMERASE CHAIN REACTION TECHNIQUE

The levels of uPAR messenger RNAs (mRNAs) in choroidal tissues were determined by reverse transcriptase–polymerase chain reaction (RT-PCR) analysis. Total RNA was extracted from tissues treated with or without laser and used to generate first-strand complementary DNA using Superscript reverse transcriptase (Gibco, Grand Island, NY). The relative level of uPAR mRNA was standardized to a coamplified invariant mRNA (18S ribosomal RNA) using standard PCR protocols. Ten microliters of each PCR was examined by agarose gel electrophoresis and ethidium bromide staining.
RESULTS

EXPRESSION OF uPAR DURING CNV

To determine the role of the uPA-uPAR system in CNV, we examined RPE-choroid complexes in the mouse model of CNV by RT-PCR analysis 14 days after laser treatment.1 The presence of uPAR mRNA was analyzed in the RPE-choroidal tissues on days 5, 10, and 14 following laser induction of CNV. The uPAR mRNA expression was detectable at all time points compared with age-matched control animals without laser treatment, which showed little to no uPAR mRNA expression in the RPE-choroidal tissues (Figure 1).

LOCALIZATION OF uPAR DURING CNV

To determine the cell types responsible for uPAR expression in this CNV model, we then examined the tissues in control and experimental animals by immunocytochemical analysis. The uPAR was localized to the endothelial lining of blood vessels within the fibrovascular tissue (Figure 2). Adjacent sections stained with an endothelium-specific antibody to CD31 confirmed that the staining of uPAR was limited to the endothelial cells within this fibrovascular complex of the laser burn area. Control sections without the primary antibody treatment did not show any uPAR staining of the laser burn area.

EFFECT OF Å6 ON CNV

The ability to inhibit the development of CNV by disruption of the uPA-uPAR system was investigated by treating animals with increasing concentrations of the Å6 peptide. The Å6 peptide, or PBS, was given intraperitoneally according to the protocol described herein. The area within the burn occupied by new disorganized vessels was obvious microscopically and appeared to decrease with Å6 treatment (Figure 3). At the highest dose of Å6, the burn area appeared to be completely devoid of new vessels. However, as seen at higher magnification (Figure 3E), there were small numbers of fluorescein isothiocyanate-conjugated dextran-labeled vessels present. Quantitation and statistical analysis of the extent of CNV in whole mounts of RPE-choroid complexes as determined by the area of fluorescein isothiocyanate–
conjugated dextran-labeled vessels following treatment indicated that there was significant inhibition of CNV in this animal model (Figure 4). The effect of Å6 on inhibition of CNV development was found to be dependent on the frequency at which Å6 was given. The inhibition was minimal and nonsignificant with the less frequent doses of 100 mg/kg once per week (P = .23). When Å6 was given at 100 mg/kg every third day, a small but significant decrease was seen in the extent of neovascularization (37%, P = .02). The most frequent dose of 100 mg/kg given every day produced the most significant degree of inhibition (95%, P < .001) (Figure 4). None of the treated mice exhibited any apparent toxic effects to the peptide during the 2-week treatment period. There were no apparent tissue toxic effects as judged by histologic analysis of the retina and choroid (not shown). In addition, there was no evidence of tissue destruction or abnormality in the choroidal vessels outside the burn areas.

**COMMENT**

Extracellular proteinases facilitate cell migration during angiogenesis by regulating cell-matrix interactions through both proteolytic and nonproteolytic mecha-

Figure 3. Å6 treatment prevents neovascularization in the laser-induced model of choroidal neovascularization (CNV). Representative images of retinal pigment epithelium–choroid whole mounts infused with fluorescein isothiocyanate–conjugated dextran 14 days after laser induction of CNV. Images are from mice treated with phosphate-buffered saline or Å6 peptide dosed at differing frequencies. The red circle roughly outlines the area of the laser burn. The fluorescence in the center of the burn area demonstrates the extent of the new vessel formation under the retina. The surrounding fluorescence represents the normal choroidal vasculature. A, Mouse treated with phosphate-buffered saline twice a day for 14 days. B, Mouse treated with the Å6 peptide, 100 mg/kg twice a day once a week. C, Mouse treated with Å6, 100 mg/kg twice a day every third day. D, Mouse treated with Å6, 100 mg/kg twice a day every day. E, A higher magnification image of the central region in section D. Only a few fluorescein isothiocyanate–conjugated dextran-labeled blood vessels could be seen in this area.
extracellular matrix components. The proteolytic activity of uPA is physiologically regulated by plasminogen activator inhibitor 1 (PAI-1), a member of the serine proteinase inhibitor (SERPIN) family. Urokinase binds with high affinity to a cell surface receptor, uPAR, present on many cell types, including endothelial cells. The interactions of uPA, uPAR, and PAI-1 can affect cell motility by localizing proteolytic activity to the cell surface and regulating the activity of specific cell-matrix adhesion receptors.

Results from the present study demonstrate that uPAR is significantly up-regulated during the development of laser-induced CNV in the mouse. The laser-induced CNV model relies on the production of a focal injury to the Bruch membrane to induce the formation of new vessels. It is conceivable that inflammatory cells initially occupy the site of the laser burn and subsequently release cytokines and growth factors, causing an up-regulation of uPAR and extracellular matrix proteins by the activated endothelial cells of the surrounding choroid. The uPAR was localized by immunocytochemical analysis specifically to the endothelial cells within the fibrovascular area of the laser burn. The surrounding normal choroidal vessels in contrast did not demonstrate any significant uPAR expression. This is consistent with the fact that uPA and uPAR are not expressed by quiescent endothelium and are expressed only during active angiogenesis.

Recently, the mRNAs of uPA and uPAR have been detected in choroidal neovascular membranes from patients with ARMD and animals with experimental CNV. Hypoxia, a known major stimulus for angiogenesis, can increase uPAR expression in endothelial cells, and uPAR is up-regulated during retinal angiogenesis. Recent evidence indicates that there may also be a possible role of inflammatory cells in the pathogenesis of retinal neovascularization. These cells may have a role in regulating uPAR expression via cytokine production. However, it is not clear whether hypoxia plays any role in the formation of CNV. It is possible that inflammatory cytokines and growth factors such as vascular endothelial growth factor may be responsible for the increased expression of uPAR in the endothelial cells during the development of CNV.

In the present study, we demonstrated that by inhibiting the uPA-uPAR system with the A6 peptide CNV can be significantly inhibited in a dose scheduling-dependent manner. A6, an 8-amino acid peptide from the connecting region of urokinase (amino acids 136-143), inhibits the uPA-uPAR interaction in a noncompetitive manner. This peptide has been shown to inhibit tumor growth and lymph node metastasis in breast cancer and glioblastoma models without any direct cytotoxic effects. The antiangiogenic activity of the A6 peptide in the tumor models has been associated with a significant decrease in the density of blood vessels in these tissues. A mechanism for this inhibition of new vessel formation was suggested to be due to a decrease in transforming growth factor β activity and expression of the vascular endothelial growth factor receptor Flk-1 as a direct or indirect result of the inhibition of the uPA-uPAR system. Alternatively, work by Czekay et al has demonstrated the requirement for uPA-uPAR interactions in the PAI-1-mediated recycling of uPAR and associated integrins that facilitates cell detachment from components of the extracellular matrix. Inhibition of the uPA-uPAR system by A6 might therefore be expected to disrupt this recycling process, causing increased cell-matrix adhesion and thus rendering the cells immotile.

Similar inhibition of retinal neovascularization with the A6 peptide in the animal model of ischemia-induced retinopathy has recently been shown in our laboratory. These data and the results from the present study together suggest that the uPA-uPAR system may be a potential therapeutic target for many types of abnormal ocular angiogenesis. The effect is independent of the initiating stimuli, since both CNV (inflammation or injury) and retinal neovascularization (hypoxia or ischemia) respond equally well to A6 treatment.

The current treatments for CNV, including conventional laser therapy and photodynamic therapy, are not optimal and have limitations. The conventional laser therapy results in scotoma, severe vision loss, and recurrences in 50% of the cases. The photodynamic therapy does not cause permanent regression of new vessels, since the vessels always grow back. This procedure requires repeated treatments to close the new vessels, and there is also collateral damage to the surrounding retina, choroid, and RPE cells. Numerous attempts have been made to inhibit ocular neovascularization using pharmacologic approaches. These approaches include inhibition of growth factor binding, integrin function, and protease activities.
Successful and effective antiangiogenic therapies may be useful as an alternative or an adjunct to the current laser treatment. The effectiveness of the A6 peptide in inhibiting CNV in the mouse without any adverse effects suggests that inhibition of the uPA-uPAR system with this peptide may be a useful alternative therapy in the management of CNV.

Submitted for Publication: July 31, 2003; final revision received April 29, 2004; accepted April 29, 2004.

Correspondence: Arup Das, MD, PhD, Department of Surgery, University of New Mexico School of Medicine, 2211 Lomas Blvd NE, Albuquerque, NM 87131 (adas@unm.edu)

Funding/Support: The study was supported in part by grant RO1-EY12604-05 (Dr Das) from the National Institutes of Health, Bethesda, Md.

Previous Presentation: This study was presented at the Annual Meeting of the Association for Research in Vision and Ophthalmology; May 7, 2003; Fort Lauderdale, Fla.

REFERENCES


