Synthetic Peptides Interacting with the 67-kd Laminin Receptor Can Reduce Retinal Ischemia and Inhibit Hypoxia-Induced Retinal Neovascularization

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The high-affinity 67-kd laminin receptor (67LR) is expressed by proliferating endothelial cells during retinal neovascularization. The role of 67LR has been further examined experimentally by administration of selective 67LR agonists and antagonists in a murine model of proliferative retinopathy. These synthetic 67LR ligands have been previously shown to stimulate or inhibit endothelial cell motility *in vitro* without any direct effect on proliferation. In the present study, a fluorescently labeled 67LR antagonist (EGF₃₃₋₄₂) was injected intraperitoneally into mice and its distribution in the retina was assessed by confocal scanning laser microscopy. Within 2 hours this peptide was localized to the retinal vasculature, including preretinal neovascular complexes, and a significant amount had crossed the blood retinal barrier. For up to 24 hours postinjection, the peptide was still present in the retinal vascular walls and, to a lesser extent, in the neural retina. Non-labeled EGF₃₃₋₄₂ significantly inhibited pre-retinal neovascularization in comparison to controls treated with phosphate-buffered saline or scrambled peptide ($P < 0.0001$). The agonist peptide (Lam β 1₉₂₅₋₉₃₃) also significantly inhibited proliferative retinopathy; however, it caused a concomitant reduction in retinal ischemia in this model by promoting significant revascularization of the central retina ($P < 0.001$). Thus, 67LR appears to be an important target receptor for the modulation of retinal neovascularization. Agonism of this receptor may be valuable in reducing the hypoxia-stimulated release of angiogenic growth factors which drives retinal angiogenesis. (Am J Pathol 2002; 160:307-313)

The inappropriate proliferation of retinal capillaries derived from pre-existing vessels (retinal neovascularization) is a significant complication of many important ocular conditions such as diabetic retinopathy, branch vein occlusions, and retinopathy of prematurity. Together

these conditions constitute major causes of blindness and yet the ability to prevent neovascularization is severely limited and is currently reliant on ablation of functional retina using laser photocoagulation or cryotherapy. The underlying basis for retinal neovascularization and the complexity of the angiogenic stimulus is becoming clearer. Vascular endothelial growth factor (VEGF) and other related angiogenic peptides are now known to have a critical role in initiating and propagating the neovascular response¹ and effective neutralization of these factors is a hopeful avenue for therapeutic intervention. However, this optimism must be tempered by the realization that many such factors are also important promoters of vascular cell survival,² therefore putative inhibitory substances would need to be carefully titrated and delivered within defined periods of the proliferative response. An alternative approach to controlling retinal neovascularization is to antagonize adhesion-dependent migration of activated endothelial cells. Agents which can block receptor-mediated interactions of migrating endothelial cells with the extracellular matrix (ECM) would be expected to preferentially target the actively proliferating retinal vessels.^{3,4}

Laminin is a major component of vascular basement membranes and is vital for endothelial cell function under physiological conditions.⁵ Cellular interaction with laminin α , β , and γ chains is achieved through a range of integrin and non-integrin receptor interactions that coordinate cellular adhesion, spreading, differentiation, and phenotypic stabilization.⁶ Among the many laminin-binding proteins, a high-affinity non-integrin laminin receptor which migrates at 67 kD, after posttranslational modification of a ~33-kD precursor protein (designated P40/37LRP),^{7,8} has been identified in vascular endothelial cells.^{9,10} This receptor (designated 67LR) binds to a cysteine-rich domain of the short arm of laminin β 1.¹¹

Tumor cell-associated 67LR has a recognized role in metastasis and tumor invasiveness.^{12,13} 67LR is also known to facilitate attachment and migration of endothelial cells and, given its positive correlation with microves-

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sel density in tumors, endothelial cell-associated 67LR is also likely to have a crucial function in tumor angiogenesis.^{13–15} In a murine model of proliferative retinopathy, 67LR was found to be highly expressed by proliferating intraretinal and preretinal new vessels.¹⁶ This was in direct contrast to the established, quiescent, retinal vasculature where expression was barely detectable.¹⁶ In addition, it has been shown that 67LR is highly expressed by proliferating microvascular endothelium during retinal development¹⁷ and that, *in vitro*, expression levels decrease significantly when these cells become contact-inhibited.⁹

It has been shown that synthetic peptides with homology to the binding site of 67LR on the laminin β 1 chain (residues 925–933 of murine β 1; sequence CPDGYIGSR) may display agonist or antagonist properties and can effectively enhance or reduce epidermal growth factor (EGF)- or laminin-stimulated endothelial cell motility, respectively.¹⁰ For example, a peptide antagonist of 67LR, derived from the murine EGF amino acid sequence 33–42 (EGF_(33–42); sequence VIGYSGDR) inhibits endothelial cell motility *in vitro*, whereas the native laminin β 1 peptide (Lam β 1_{925–933}) acts as an agonist, stimulating endothelial cell motility.^{10,14,18} Thus, 67LR represents a potentially useful therapeutic target for modulating retinal neovascularization and to test this we used synthetic peptides which display either agonist or antagonist properties in a murine model of hypoxia-induced proliferative retinopathy.

Materials and Methods

Synthesis of Peptide Analogues

The following synthetic peptides were used in the experiments: a decapeptide from the C-loop of murine EGF (EGF_{33–42}) (acetyl-C-(S-Acm)-VIGYSGDR-C(S-Acm)-NH₂), a scrambled, control peptide (based on a randomized peptide from the EGF_{33–42} sequence; acetyl-IDC-(S-Acm)-YGC-(S-Acm)-RSVG-NH₂) and a nonapeptide Lam β 1_{925–933} (amino acid sequence: CPDGYIGSR), corresponding to the binding region for 67LR on laminin β 1 chain (amino acid residues 925–933) (Lam β 1_{925–933}). For fluorescein isothiocyanate (FITC) labeling of EGF_{33–42}, the carboxyfluorescein (Fluka, Dorset, UK) was coupled to the amino-terminal cysteine using fluorenylmethycarbonyl (Fmoc) chemistry. All peptides were synthesized on a model 432A peptide synthesizer (Applied Biosystems, Warrington, UK), using standard solid-phase Fmoc procedure. They were then purified using reverse-phase high performance liquid chromatography (HPLC) and the purity confirmed by capillary electrophoresis, automated amino acid analysis, and electrospray mass spectrometry.

Animal Model and Experimental Groups

The studies adhered to the Association for Research in Vision Ophthalmology statement for the use of Animals in Ophthalmic and Vision Research. Oxygen-induced retinopathy (OIR) was induced in C57BL/J6 mice according

to a protocol which has been described previously.¹⁹ Briefly, litters of 7-day-old (P7) pups and their nursing dams were exposed to 75% oxygen for 5 days. The flow of humidified medical grade oxygen was controlled by a gas oxygen controller (PROOX model 110; Reming Bioinstruments, Redfield, NY). On postnatal day 12 (P12) the mice were returned to ambient oxygen. Body weights were recorded on P7 and daily from P12 to P20 to ensure that there was no serious growth retardation.

FITC-EGF_{33–42} was administered intraperitoneally (i.p.) into hypoxia-exposed ($n = 5$) and normoxia control mice ($n = 4$) at P20. At 2, 6, and 24 hours postinjection the eyes were enucleated and fixed in 4% paraformaldehyde (PFA). The anterior segment, lens, vitreous, and hyaloid were removed and the posterior eye cup was subjected to four radial full-thickness cuts and incubated for 16 hours at 4°C in phosphate-buffered saline (PBS) containing 0.5% Triton X-100 (TX-100). The retinal vasculature was then localized through labeling with biotinylated BSII lectin (purified from *Griffinia simplicifolia*, Sigma Chemical Company) and subsequently, streptavidin-Texas Red (Dako Ltd.). The eye cups were then flat mounted and fluorescence was localized using a confocal scanning laser microscope (CSLM). Kidneys, liver, and brain from these animals were also harvested and fixed before frozen sections were prepared and viewed by CSLM.

Non-labeled EGF_{33–42} and Lam β 1_{925–933} in two different concentrations (10.0 and 2.0 mg/kg/day, diluted in PBS) were administered i.p. daily from P12 to P19 to a minimum of 12 pups per group. Scrambled peptide was used in the highest dose (10.0 mg/kg/day) only.

Each litter of hypoxia-exposed mice was divided into two groups, one of which was a peptide-treated group and the other was designated to be injected with either PBS or scrambled peptide and was used as the control for each experiment. Both groups stayed together throughout the whole experiment (P0 to P20) to eliminate a possible difference in growth rate originating from different nursing conditions. Routinely, two pups from a litter were killed upon returning to the room air (P12) to check for hypoxia-mediated closure of the central retinal capillaries and the rest of the litter 8 days later (P20). Before sacrifice, the mice were deeply anesthetized by intraperitoneal injection as previously described¹⁶ and given 0.15 ml of FITC-dextran (50 mg/ml in PBS) via the left ventricle (fluorescein isothiocyanate dextran, MW: 2×10^6 ; Sigma-Aldrich). The eyes were enucleated, fixed in 4% paraformaldehyde solution in 0.1 mol/L phosphate buffer for 18 hours, and then washed in PBS. The retinas were carefully dissected and flat mounted on microscope slides in a Maltese cross configuration.

Quantification of Neovascular Response

Flat-mounted FITC-dextran perfused retinas were imaged on a BioRad MicroRadian confocal scanning laser microscope fitted to an Olympus BX60 fluorescence microscope with a 4× plan-apochromatic objective. For

comparative analysis, the retinal angiographic images were always orientated with the optic nerve at the center of the field of view. The angiographic analysis was conducted according to a novel method (Gebarowska D, Stitt AW, Mahon A, Nelson J, Gardiner TA, submitted for publication) which displayed each digital angiographic image with a superimposed 64-square grid (8×8 squares) corresponding to a real area of 9.95 mm^2 . Each grid square, equivalent to 0.155 mm^2 of retinal area, was analyzed and annotated with on-screen letters which specifically recorded and quantified normally vascularized retina, residual ischemic retina at P20, and the vasoproliferative response as registered by preretinal neovascularization and intraretinal tufts of new vessels. A computer program using the classification described below was designed to assist with the retinal angiogram analysis and classified vessels according to the following: E, empty; N, normally vascularized retina; I, ischemic non-perfused retina; T, neovascular tufts; O, non-vascularized far periphery; V, tortuous vessels; and U, unidentifiable. "Empty" represented those areas of an image corresponding to the expansion of the four radial cuts applied in the flat-mounting procedure. An operator familiar with the relevant angiographic morphology applied the letter codes. The annotation procedure allowed for the recording of different features within any given grid square, ie, coding a square by more than one letter, which was usually necessary as a retinal area of 0.155 mm^2 may be characterized by several angiographic features. The program was able to quantify all possible letter combinations and calculate the total retinal areas displaying the particular morphologies. The total areas characterized by each of the designated features were expressed in mm^2 and as percentages of a total analyzed retinal area. The program performed simple summary statistics of an analyzed image and the data files were transferred to other programs for further analysis and display where the data were compared by one-way analysis of variance.

Since litter size was variable and there was always control and experimental animals within litters, the number of mice in each experiment was variable. However, for all experiments there was a minimum of 12 eyes analyzed from 6 mice and a maximum of 24 eyes from 12 mice per group.

Animal Growth, Organ Weights, and Histological Analysis

Mice body weights were recorded on P7 and daily from P12 to P20 and compared between two groups of the same "nursery" litter. At the end of the experiments hearts, livers, lungs, kidneys and spleens were collected, weighed, fixed in 4% paraformaldehyde, and processed for light microscopic examination after hematoxylin and eosin staining. An experienced pathologist evaluated the organs for alterations in morphology.

Figure 1. Hypoxia-induced proliferative retinopathy in the mouse model. **A:** Normal murine retinal vasculature showing perfusion of central retina as assessed by confocal scanning laser microscopy of angiograms (Magnification, $\times 40$). Following exposure of hypoxia (75% for 5 days between P7 and P12) there is closure of the central capillary beds (**B**) which is followed by preretinal neovascularization up to P21 in response to retinal hypoxia in normal oxygen conditions (**C**; **inset**, $\times 200$ magnification of neovascular frond). **Arrows** depict preretinal neovascularization; * indicates avascular regions at P12. Original magnification, $\times 40$.

Results

In comparison to retinas from mice which had not been pre-exposed to hypoxia, experimental mice consistently exhibited closure of the central retinal vasculature at P12 (compare Figure 1, A and B). By P20 there was evidence

Figure 2. Localization of FITC-labeled EGF₃₃₋₄₂ in the murine retina. Throughout this figure the red channel image is lectin staining; green channel image is FITC-EGF₃₃₋₄₂ and the largest image is a red/green merged image. **A:** In normoxia-exposed control mice (P20) i.p.-injected FITC-EGF₃₃₋₄₂ was localized to the retinal vasculature of the nerve fiber layer within 2 hours. There was evidence that the peptide had traversed the blood retinal barrier as depicted in green fluorescence within the cytoplasm of the ganglion cells. Original magnification, $\times 800$. **B:** In hypoxia-exposed P20 mice the FITC-EGF₃₃₋₄₂ was localized to the intra and preretinal vessels 2 hours after injection. Hyperfluorescent areas may indicate an accumulation of the peptide within the neovascular fronds (arrows). Original magnification, $\times 400$. **C:** Within the inner plexiform layer the green fluorescence of the peptide analogue was largely localized to the capillaries of the deep capillary plexus although there was also evidence of some leakage into the neural retina. Original magnification, $\times 400$. **D:** After 24 hours postinjection of FITC-EGF₃₃₋₄₂ there was still green fluorescence in the neovascular fronds (arrow) although this was less widespread in the surrounding vasculature than after 2 hours. There was still diffuse fluorescence in the neural retina. Original magnification, $\times 600$.

of a proliferative response after return to normoxic conditions, with fronds of new retinal vessels being visible in the fluorescein angiograms (Figure 1C).

CSLM analysis 2 hours after FITC-EGF₃₃₋₄₂ injection of P20 control mice demonstrated that this peptide reached the retinal vasculature and had apparently crossed the blood-retina barrier (Figure 2A). When labeled peptide was introduced into hypoxia-exposed mice there were intense accumulations of this labeled peptide within the neovascular fronds at 2 and 6 hours (Figure 2B). FITC was also delineated to the superficial and deep capillary beds (Figure 2C). At 24 hours postinjection there was considerably less FITC label in the preretinal vasculature and it appeared to be largely basement membrane associated in the intraretinal vessels (Figure 2D). In sections of other organs FITC-EGF₃₃₋₄₂ was apparent in the profiles of blood vessels with diffuse fluorescence apparent in the extravascular space. In kidney there was some evidence of diffuse fluorescence in the tubule epithelium (data not shown).

Evaluation of the fluorescein angiograms coupled with a computerized analysis approach allowed quantification of the absolute areas of normally vascularized retina,

avascular tissue, and areas of preretinal neovascularization. It was found that the retina of P12 mice with OIR showed total non-perfusion in the regions of the central retinal capillary beds, estimated at a mean area of $5.43 \pm 0.46 \text{ mm}^2$ (corresponding to $66.5 \pm 6.2\%$ of the total retinal area). The retina of hypoxia-treated P20 mice showed a significant preretinal neovascular response, which extended from the surviving peripheral vasculature, and variably from the optic disk to cover previously ischemic central retina (Figure 1C). The mean area of retina showing preretinal neovascularization in these animals measured $4.94 \pm 0.61 \text{ mm}^2$ (equivalent to $59.1 \pm 7.3\%$ of the total retinal area).

Evaluation of retinas from mice treated from P12 with mEGF₃₃₋₄₂ (Figure 3A) showed a clear inhibition of neovascularization compared with a scrambled peptide-injected control group (Figure 3B). At the same time, EGF₃₃₋₄₂ treatment had no effect on quiescent, non-proliferative retinal vessel density. On quantification, there was a significant reduction in the occurrence of preretinal vessels in mice treated with EGF₍₃₃₋₄₂₎ at either 2.0 ($P < 0.005$) or 10.0 mg/kg/day ($P < 0.001$) compared with controls (Figure 3C). The lowest concentration

Figure 3. Modulation of proliferative retinopathy by the 67LR peptide antagonist EGF₃₃₋₄₂. Treatment of mice with EGF₃₃₋₄₂ (daily from P12 to P20; 2 or 10 mg/kg, i.p.) caused a significant reduction in neovascularization above the internal limiting membrane (**A**; compare with Figure 2B). Treatment with a scrambled peptide sequence lead to no significant reduction in preretinal neovascularization (**B**; compare with Figure 1C). Original magnification, $\times 40$. **C**: Treatment of mice with EGF₃₃₋₄₂ at 2 or 10 mg/kg/day (E₂; E₁₀) resulted in a significant reduction in neovascularization when compared to non-treated control (NC) or scrambled peptide (SC) groups ($n > 12$ mice/group) (* $P < 0.005$; ** $P < 0.001$). **Bars** show SEM.

proved to be equally effective at reducing preretinal neovascularization in hypoxia-exposed mice at P20.

P20 mice which had been treated with Lam β 1₉₂₅₋₉₃₃ for 8 days demonstrated a significant reduction in retinal neovascularization at the highest dose of 10 mg/kg/day ($P < 0.001$ when compared to control, hypoxia-exposed mice) (Figure 4). Full angiographic analysis of this series of experiments revealed marked differences compared with the appearance of EGF₃₃₋₄₂-treated retinas. In Lam β 1₉₂₅₋₉₃₃-treated animals there was a marked increase in the central retinal capillary density in comparison to EGF₃₃₋₄₂ and control retinas (compare Figure 4, A and B with Figure 1C and Figure 3A). Quantitative analysis revealed a significant reduction in retinal ischemia and an increase in intraretinal revascularization of the ischemic central retina ($P < 0.001$) with a tendency toward "normality" when compared to scrambled peptide-treated mice or hypoxia controls (Figure 4C).

During treatments, neither EGF₃₃₋₄₂, Lam β 1₉₂₅₋₉₃₃, or the scrambled peptide had a significant influence on mouse body weight when compared to hypoxia-treated control mice. On postmortem examination of other organs it was apparent that the peptides also did not influence weights of liver, lungs, or spleen. However, with EGF₃₃₋₄₂ treatments there was a significant increase in heart

Figure 4. Treatment with the 67LR agonist Lam β 1₉₂₅₋₉₃₃ modulates retinal ischemia and neovascularization. Similarly to EGF₃₃₋₄₂ (Figure 3), Lam β 1₉₂₅₋₉₃₃ also caused a reduced neovascularization at 2 mg/kg/day (**A**) and 10 mg/kg/day (**B**). However, there was a concomitant increase in intraretinal revascularization and increase in retinal vascular "normality" (compare Figure 4, A and B with Figure 3, A and B). Original magnification, $\times 40$. **C**: Lam β 1₉₂₅₋₉₃₃ at the highest dose produced a reduction in neovascularization comparable to that of EGF₃₃₋₄₂ (** $P < 0.001$). There was a significant increase in intraretinal revascularization of the central retina with accompanying reduction in ischemia (** $P < 0.001$). Generally there was a tendency toward "normality" when compared to hypoxia-exposed scrambled peptide-treated control (C) mice. **Bars** show SEM.

weight ($P < 0.033$) and decrease in kidney weight ($P < 0.038$) when compared to controls. On pathological assessment of the organs there was no apparent influence of any of the peptide treatments on tissue organization or qualitative vessel density (data not shown).

Discussion

A previous study by Stitt et al¹⁶ has shown that 67LR is up-regulated in proliferating retinal vasculature during hypoxia-induced proliferative retinopathy. Furthermore, expression of this receptor is also increased during murine postnatal retinal vascular development.¹⁷ Following on from this, the present investigation has demonstrated that the 67LR antagonist EGF₃₃₋₄₂ can achieve high concentrations in the retinal vasculature when injected i.p. and such treatment significantly inhibits preretinal neovascularization in the murine model of OIR. It is apparent, therefore, that this laminin receptor plays a critical role in proliferative retinopathy, probably by preventing endothelial cell migration which is an essential component of

the angiogenic process. This is supported by *in vitro* studies using EGF_(33–42) which showed that this peptide significantly inhibits 67LR-mediated endothelial cell motility and migratory capacity.¹⁰ Most proliferating cells require the expression of adhesion molecule receptors to interact with their underlying substratum.¹³ 67LR, by virtue of its high affinity for laminin, appears to have an important role in rapidly anchoring cells to the substratum during cell proliferation before integrin-mediated cell spreading.^{15,20}

It has been shown that once proliferation responses reduce (eg, as a result of contact inhibition *in vitro*), endothelial cells, including retinal microvascular endothelium, markedly down-regulate their expression of 67LR.^{9,16} Indeed, it has been shown that 67LR expression is reduced or absent on quiescent intraretinal capillaries in comparison to actively proliferating vessels.¹⁶ In the current study, EGF_{33–42} had no apparent influence on quiescent, non-proliferative retinal vessel density as evidenced by the persistence of the central retinal ischemia and no change at the peripheral capillary plexi or large central retinal vessels. Being a cell surface receptor which is comparatively highly expressed by proliferating endothelial cells makes 67LR an promising target for treatment of retinal neovascularization and perhaps other angiogenic diseases such as metastatic cancer.

That Lamβ_{1925–933} caused a reduction in neovascularization comparable to that of EGF_{33–42} was unexpected, as this peptide has been previously shown to function as an agonist of 67LR-mediated endothelial cell migration *in vitro*.¹⁰ However full angiographic analysis revealed differences compared with the appearance of EGF_{33–42}-treated retinas in that there was a clear tendency toward “normality” when compared to hypoxia-exposed control mice. The data suggests that in this model system the Lamβ_{1925–933} nonapeptide is acting as a partial 67LR agonist, in that it promotes endothelial cell migration and revascularization of the ischemic neural retina. Significantly, there was also a decrease in preretinal neovascularization which is most likely due to the reduction in retinal ischemia and hence hypoxia-mediated expression of potent angiogenic stimuli such as VEGF.

This is the first report on the ability of a fragment of the β chain of laminin-1 to cause a significant revascularization event in any tissue. However, previous studies have suggested that peptide fragments based around the YIGSR motif can act as 67LR agonists and promote endothelial cell sprouting, adhesion, and tube formation *in vitro*.^{14,21} The present results resemble those obtained from studies of the bioactivity of the peptide SIKVAV (a motif derived from the α chain of laminin-1) which can also promote angiogenesis *in vitro* and *in vivo*^{22,23} and can appreciably reverse hind limb ischemia by augmenting capillary recanalization.²⁴ It is possible that agonist stimulation of the retinal vasculature with Lamβ_{1925–933}-based peptides may allow more rapid cell spreading and thus enhance the endothelial angiogenic phenotype.

Reversal of retinal ischemia would be a highly effective therapeutic option for many important retinal disorders such as diabetic retinopathy and retinopathy of prematurity. Agonism of 67LR and other similar receptors may lead to a reversal of retinal vascular insufficiency through

promotion of intraretinal angiogenic activity without leading to uncontrolled proliferation on the retinal surface, a phenomenon which carries high risk of vitreal bleeding and tractional retinal detachment. In many disease states, a strategy of ischemia reversal has obvious advantages over agents which directly inhibit endothelial cell proliferation since it offers the option to repair tissue damage which is the primary stimulus for neovascularization. The potential uses of such pro-angiogenic peptides warrants further study.

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