Experimental Eye Research 88 (2009) 79–91

Contents lists available at ScienceDirect

Experimental Eye Research

journal homepage: www.elsevier.com/locate/yexer

Differences in the temporal expression of regulatory growth factors during choroidal neovascular development

Wenzheng Hu [a,b], Mark H. Criswell [a,b,*], Shao-Ling Fong [a], Constance J. Temm [b], Gangaraju Rajashekhar [b], Tammy L. Cornell [a], Matthias A. Clauss [b]

[a] Department of Ophthalmology, Indiana University School of Medicine, 702 Rotary Circle, Indianapolis, IN 46202, USA
[b] Indiana Center for Vascular Biology and Medicine, Indiana University School of Medicine, Indianapolis, IN 46202, USA

ARTICLE INFO

Article history:
Received 29 January 2008
Accepted in revised form 14 October 2008
Available online 1 November 2008

Keywords:
choroidal neovascularization
vascular endothelial growth factor
basic fibroblast growth factor
hepatocyte growth factor
laser photocoagulation
angiogenesis
anastomoses
neovascular hyperpermeability

ABSTRACT

Although the roles of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and hepatocyte growth factor (HGF) in pathologic neovascularization have been well characterized in certain tissues, their particular functions and expression patterns in choroidal neovascularization (CNV) have not been clearly established. After localized laser trauma to Bruch's membrane to induce CNV development, the temporal changes in mRNA and protein expression of these 3 cytokines were documented and compared histologically to areas of immunofluorescence, the proliferation of endothelial cells, neovascular development, and temporal changes in vascular permeability. Changes in mRNA and protein levels of bFGF and HGF occurred quickly and reached peak expression within hours. This activity corresponded in time to intense and localized immunofluorescence for these cytokines within the choriocapillaris within laser lesion sites. During this same initial time period, mRNA upregulation of VEGF occurred, primarily within the neural retina and this expression corresponded to intense immunolabeling of Müller cells immediately adjacent to the lesion sites. By 3 days after lasering, increased $VEGF_{164}$ protein expression was measurable, whereas early neovascular development histologically corresponded to HGF and bFGF mRNA expansion into the developing choroidal neovascular membrane (CNVM). At 7 days, CNV expansion, maturation, and increased vascular permeability corresponded to peak VEGF mRNA and protein expression and to immunofluorescence of the CNVM. Differences also occurred in the expression of precursor and activated isoforms of these cytokines in the retinal pigment epithelium/choroid as compared to those in the retina. These molecular and immunocytochemical results suggest that bFGF and HGF may be important as initial regulators neovascularization in this CNV model; whereas VEGF may be important during later phases of angiogenesis and neovascular hyperpermeability.

1. Introduction

Choroidal neovascularization (CNV) is an important component of posterior segment diseases and results from complex interactions between angiogenic regulators that promote and/or inhibit endothelial cell differentiation, proliferation, and migration, as well as vascular maturation, stabilization, maintenance and permeability. Numerous angiogenic regulators participate in CNV development, including three multifunctional growth factors that possess the potential to affect multiple aspects of the angiogenic process: vascular endothelial growth factor (VEGF) (Frank et al., 1996; Kvanta et al., 1996; Spilsbury et al., 2000; Lip et al., 2001; Wang et al., 2003), basic fibroblast growth factor (bFGF or FGF-2) (Frank et al., 1996; Ogata et al., 1996), and hepatocyte growth factor (HGF or scatter factor) (Grierson et al., 2000).

VEGF functions as a potent mitogen in embryogenesis and in tumorigenesis, but its specific angiogenic roles in CNV have been largely based on inferential evidence. In age-related macular degeneration (AMD) patients, elevated VEGF expression has been demonstrated in patient vitreous samples and immunostained ocular tissues from preexisting neovascularization, although the exact relationship of VEGF to CNV angiogenesis remains under investigation. VEGF signaling also occurs after laser-induced rupture of Bruch's membrane evokes CNV (Kwak et al., 2000). Subretinal or vitreal infusion of exogenous VEGF can evoke experimental CNV, but then so can the over expression of other angiogenic regulators, including pigment epithelium derived factor (PEDF) which typically demonstrates antiangiogenic properties (Apte et al., 2004).

* Corresponding author. Department of Ophthalmology, Indiana University School of Medicine, 702 Rotary Circle, Indianapolis, IN 46202-5175, USA. Tel.: +1 317 274 2836; fax: +1 317 274 2838.
E-mail address: mcriswel@iupui.edu (M.H. Criswell).

0014-4835/$ – see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.exer.2008.10.014

VEGF upregulation can occur in endothelial cells of forming capillaries as a consequence of increased bFGF expression (Seghezzi et al., 1998; Shen et al., 1998; Wada et al., 1999). VEGF release from aging and damaged blood vessels may be one of the signaling molecules for circulating endothelial cells (CECs) and endothelial precursor cells (EPCs) during early postnatal vasculogenesis; however, bFGF is more potent than VEGF as a mitogenic activator for CEC proliferation (Zubilewicz et al., 2001; Wang et al., 2005). Nevertheless, bFGF is not considered able to singularly evoke CNV development (Tobe et al., 1998; Ishida et al., 1999; Campochiaro, 2000; Nagineni et al., 2003; Rosenthal et al., 2005; Hoffmann et al., 2006). While bFGF may be one of the initial inducers of endothelial cell proliferation, VEGF, in parallel or subsequently, is another mediator of this process.

HGF is a plasminogen-derived, multifunctional heterodimeric polypeptide produced by mesenchymal cells and it activates cells that express the Met tyrosine kinase receptor (Bottaro et al., 1991; Rubin et al., 1993). HGF can elicit mitogenic, motogenic, and morphogenic effects in various epithelial and endothelial cell types (Bussolino et al., 1992; Rubin et al., 1993; Nakamura et al., 1995; Matsumoto and Nakamura, 1996). HGF can mediate epithelial mesenchymal transition (Tsarfaty et al., 1994), the formation of vascular tubules and lumens (Montesano et al., 1991; Tsarfaty et al., 1992), and can independently and sufficiently induce and promote angiogenesis (Rosen et al., 1993a; Silvagno et al., 1995; Aoki et al., 2000; Taniyama et al., 2001a,b). HGF also can operate independently in mediating intraocular angiogenesis (Grierson et al., 2000). HGF signaling for retinal microvascular endothelial cell growth and migration is through the activation of PKC and phosphatidylinositol-37-kinase (PI3K) pathways, inducing MAPK phosphorylation (Cai et al., 2000). VEGF also can activate PKC, MAPK, and PI3K pathways (Xia et al., 1996). Although MAPK phosphorylation and mitogenesis can be blocked by an anti-VEGF antibody, HGF-induced MAPK phosphorylation and early HGF-induced mitogenesis neither require nor are affected by VEGF (Sengupta, S., et al., 2003). Therefore, HGF and VEGF are considered to operate in parallel and may possibly evoke synergistic actions during angiogenesis, although under certain circumstances HGF also may function as a VEGF inhibitor (Gerritsen, 2005).

Although the roles of VEGF, bFGF, and HGF in developmental, normal physiologic and pathologic neovascularization have been well characterized in certain tissues and organs of the body, their particular functions and expression patterns in CNV actually have not been well established. Using the rat CNV model, this study evaluated temporal changes in messenger RNA (mRNA) and protein expression of these three growth factors in both retinal pigment epithelial (RPE)/choroidal and retinal tissues. These temporal expression patterns were compared histologically to areas of immunofluorescence, the proliferation of endothelial cells, neovascular development, and changes in vascular permeability in developing CNV sites.

2. Materials and methods

2.1. Animals

Eighty-four male adult (~250 g) Brown-Norway rats (Charles River Laboratories, Wilmington, MA) were used in this study. All procedures were performed with strict adherence to guidelines for animal care and experimentation prepared by the Association for Research in Vision and Ophthalmology and by the Indiana University Institutional Animal Care and Use Committee. For all procedures, animals received intramuscular (IM) ketamine at 50 mg/kg, xylazine at 5 mg/kg and acepromazine at 1 mg/kg. Topical 1% tropicamide and 2.5% phenylephrine were administered for pupillary dilation and accommodative arrest.

2.2. Laser-induction of CNV

Animals received focal laser photocoagulation to induce CNV formation as described previously (Hu et al., 2005). Briefly, a series of 8 photocoagulation sites were concentrically placed around the optic disk using a diode laser (OcuLight GL, Iris Medical Instrument, Mountain View, CA) of 532 nm wavelength, 0.05 s duration, 75 μm spot size, and 120 mW power. Although not identical, this well-documented and reliable laser trauma CNV model exhibits many salient pathologic and molecular features of neovascularization clinically found in AMD.

2.3. Tissue processing for traditional mRNA and protein assessments

At designated times after the induction of laser photocoagulation sites (30 and 90 min, 6, 24 and 72 h, 1 and 4 weeks for protein and traditional mRNA analyses plus 60 min, 12 h and 2 weeks for protein analyses), eyes were enucleated from euthanized animals. Nonlasered eyes also were harvested as normal controls for both procedures. Retinal and RPE/choroidal tissues were harvested separately under dissection microscopy. Briefly, the anterior segment was removed surgically, along with as much vitreous as possible. The retina was gently detached from the choroid. The RPE/choroid complex then was peeled away using surgical blades. The tissues from 16 rats were placed immediately in RNAlater solution (Qiagen; Valencia, CA) for RNA isolation. Tissues from another 22 rats were frozen immediately at −80 °C for further protein extraction. Samples from both eyes of each animal were combined to obtain the necessary RNA and protein material for analysis at each time point.

2.4. Reverse transcription polymerase chain reaction (RT-PCR) measurements

For traditional RT-PCR measurements, total RNA was isolated from retinal and RPE/choroidal tissues using the RNeasy Mini Kit with DNAase I on-column digestion (Qiagen, Valencia, CA) and following the manufacturer's protocol. Each sample of 2 μg total RNA was reverse transcribed into 20 μl cDNA using the Superscript II cDNA synthesis Kit (Invitrogen, Carlsbad, California) and oligo $(dT)_{18}$ primers (Ambion Inc., Austin, Texas). PCR was performed with 2 μl cDNA using Platinum Taq DNA polymerase (Invitrogen) in the following conditions: starting with one time of 3 min at 94 °C, then denaturing at 94 °C for 45 s, annealing at 55 °C for 45 s, and extending at 72 °C for 60 s, total 32 cycles. The primers were: 5′-CCA TGA ATT TGA CCT CTA TG-3' (sense) and 5'-CTG TAA CCT TCT CCT TGG CC-3' (anti-sense) for HGF; 5'-AGG CTG CAC CCA CGA CAG AA-3' (sense) and 5'-CTT TGG TCT GCA TTC ACA TC-3' (anti-sense) for VEGF; 5'-GGC TTC TTC CTG CGC ATC CA-3' (sense) and 5'-GCT CTT AGC AGA CAT TGG AAG A-3′ (anti-sense) for bFGF. For each experiment, β-actin was used as a control gene. All PCR products were electrophoresed on a 1.5% agarose gel. The appropriate product size was determined by comparison with a DNA ladder. The image was captured by the ChemiDoc XRS system (Bio-RAD Laboratories, Hercules, California) and processed with Quantity One analysis software. The intensity of bands obtained by this semi-quantitative method were then exported to an excel spreadsheet where values were expressed relative to the internal β-actin control. Two independently obtained tissue samples were analyzed 3 times for each time point to ensure reproducibility and reliability of the results.

For quantitative real-time qRT-PCR measurements of mRNA expression (two-step method: denature, annealing/amplication), total RNA was isolated from retinal and RPE/choroidal tissues from lasered eyes of 3 rats at designated time points (30 and 60 min, 6,