



Vascular Endothelial Growth Factor in the Vitreous of Proliferative Diabetic Retinopathy Patients: Chasing a Hiding Prey?

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Vascular endothelial growth factor (VEGF) exerts a pathogenic role in proliferative diabetic retinopathy (PDR), a major cause of blindness in the working-age population. Accordingly, anti-VEGF drugs represent a widespread approach in PDR therapy (1). However, even though VEGF is present at picomolar/low nanomolar concentrations in the PDR vitreous as assessed by ELISA (2), high-affinity VEGF-blocking drugs are administered intravitreally at micromolar concentrations and provide a poor response in a significant percentage of patients with PDR (3). Pharmacokinetic considerations, presence of other bioactive molecules, and/or a reduced accessibility of vitreal VEGF may explain, at least in part, the partial efficacy of anti-VEGF drugs and their extremely high drug-to-target stoichiometric ratio. On these bases, we reevaluated the actual concentration of VEGF in PDR vitreous and its accessibility by VEGF traps.

With this aim, we established a near-quantitative VEGF Western blot (WB) assay (Fig. 1A) using doses of recombinant VEGF (rVEGF) between 5.0 and 17.5 ng per sample that were confirmed by ELISA. Then WB and ELISA were used in parallel to assess VEGF levels in PDR vitreous samples obtained by pars plana vitrectomy from 16 patients with type 2 diabetes (eight males, eight females;

mean \pm SD age: 66 ± 11 years, diabetes duration: 11 ± 6 years). As shown in Fig. 1B, ELISA dramatically underestimates the concentration of vitreal VEGF when compared with WB (mean \pm SEM 0.23 ± 0.09 ng/mL vs. 516.4 ± 166.8 ng/mL for ELISA VEGF and WB VEGF, respectively), with high variability in the ELISA-to-WB VEGF concentration ratio among the samples tested. Moreover, the capacity of ELISA to detect doses of rVEGF between 50 and 500 ng/mL was inhibited >99% by addition of PDR vitreous during the assay. Accordingly, recombinant forms of the decoy soluble VEGF receptors (sVEGFRs) 1 and 2, both present in PDR vitreous (4), inhibit the detection of 100 pg/mL rVEGF by ELISA with 50% inhibitory dose (ID₅₀) ~ 3.0 ng/mL and ~ 650 ng/mL, respectively. Together, these data indicate that vitreal components may contribute to the formation of noncovalent ELISA-masking VEGF complexes.

The limited accessibility of vitreal VEGF was confirmed when different VEGF-blocking drugs were evaluated for their capacity to inhibit the sprouting of human endothelial cell spheroids triggered by rVEGF or by a 1:4 dilution of the PDR vitreous (5). To this end, we used two pools of PDR vitreous from five and three patients with type 2 diabetes, respectively (six males, two females; mean \pm SD

age: 61 ± 17 years, diabetes duration: 16 ± 6 years). As shown in Fig. 1C, bevacizumab, ranibizumab, and aflibercept inhibit the activity of 30 ng/mL rVEGF with ID₅₀ ~ 1.0 nmol/L and that of PDR vitreous pools with ID₅₀ ~ 10 μ mol/L, a concentration similar to that achieved after intravitreal treatment in patients with PDR (~ 2 – 5 μ mol/L).

In the endothelial sprouting assay, rVEGF is active at concentrations ≥ 5.0 ng/mL (5). In contrast, rVEGF induced a significant biological response only at doses ≥ 500 ng/mL when tested in the presence of a 1:4 dilution of vitreous samples containing levels of vitreal VEGF below the limits of detection by ELISA and WB. No inhibition was observed for the activity of a mix of proangiogenic factors (platelet-derived growth factor subunit B [PDGF-B], stromal cell-derived factor 1 α [SDF-1 α], interleukin 8 [IL-8], fibroblast growth factor 2 [FGF2], hepatocyte growth factor [HGF], and erythropoietin, 4.0 ng/mL each). VEGF-masking binders and angiogenesis inhibitors, including sVEGFRs, may explain the inhibitory effect exerted by these vitreous samples on rVEGF.

In conclusion, VEGF is hidden in PDR vitreous and present at concentrations 10^3 - to 10^4 -fold higher than those usually reported. This is due to noncovalent VEGF-masking complexes present at various levels in the vitreous of patients with

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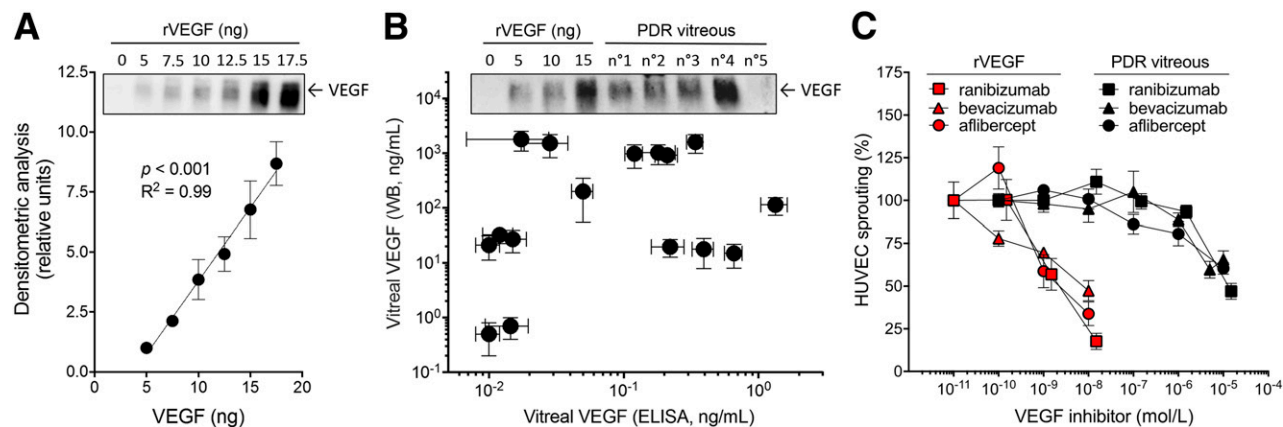


Figure 1—Quantification and neutralization of VEGF in PDR vitreous. **A:** Increasing amounts of rVEGF were run on 10% SDS-PAGE gels under nonreducing conditions. Then optical density of the immunoreactive VEGF bands (inset) was evaluated and data from five independent experiments were plotted on the same graph. **B:** VEGF levels in 16 PDR vitreous samples were evaluated by WB (10–100 μ L/sample, detection limit 0.5 ng/mL) and ELISA (25–100 μ L/sample, detection limit 10 pg/mL) in three independent experiments. Inset shows representative image of a WB performed on five vitreous samples (n°1–n°5) (10 μ L/sample) in parallel with increasing amounts of rVEGF. **C:** Human umbilical vein endothelial cell (HUVEC) spheroids embedded in fibrin gel (n = 50) were incubated with rVEGF (30 ng/mL, red symbols) or with a pool of five PDR vitreous samples (1:4 dilution, black symbols) in the presence of increasing concentrations of VEGF inhibitors. Formation of radially growing sprouts was evaluated after 24 h of incubation. Similar results were obtained with a second pool of three PDR vitreous samples (data not shown).

PDR, as indicated by the different ELISA-to-WB VEGF concentration ratios measured in the samples tested. These complexes limit the detection of vitreal VEGF by ELISA antibodies but instead are unmasked by the protein-denaturing WB procedure. Accordingly, micromolar concentrations of anti-VEGF drugs are required to neutralize the activity of VEGF in PDR vitreous. As shown by our data, decoy sVEGFRs, as well as other as yet unidentified proteinaceous/polysaccharide components, may be part of this “VEGF-masking interactome.” Notably, the presence of VEGF in vitreous exosomes has been recently hypothesized (6). Preliminary experiments suggest that VEGF may slowly dissociate in vitro from noncovalent hiding complexes following a prolonged incubation of PDR vitreous at 37°C.

Various publications attempt to correlate vitreal levels of VEGF to different clinical features in patients with PDR. Our findings suggest that the studies based on ELISA assessment of VEGF in the vitreous

should be critically evaluated for a better understanding of the mechanism of PDR. In addition, novel strategies aimed at unmasking vitreal VEGF may increase the potency and efficacy of VEGF blockers, with therapeutic implications for patients with PDR.

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