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VEGF but not P*l*GF disturbs the barrier of retinal endothelial cells

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ABSTRACT

Elevated permeability of retinal endothelial cells (REC), as observed in diabetic retinopathy (DR), is induced by extended exposure to ≥25 ng/ml vascular endothelial growth factor A_{165} ($VEGF_{165}$) for up to 3 d and this effect is more pronounced when equimolar amounts of basic fibroblast growth factor (bFGF) and insulin-like growth factor (IGF-1) are present. Down-regulation of the tight-junction protein claudin-1 and its loss from the plasma membrane is associated with induced higher permeability, whereas other tight-junction proteins (e.g. claudin-3, claudin-5, ZO-1) show only subtle changes in our experimental setting. Using immortalized bovine REC (iBREC) as a well-established model, we investigated effects of other members of the VEGF family, i.e. $VEGF_{121}$, placental growth factor (P*l*GF-1 and P*l*GF-2) and viral VEGF-E which activate different sets of VEGF receptors, on barrier function after extended treatment: iBREC were incubated with 1–100 ng/ml of the growth factors for up to 2 days before barrier function was assessed by measuring transendothelial resistance (TER). Presence of TJ-proteins was determined by western blot analyses and immunofluorescence staining. Similar experiments were performed to evaluate whether the primary actions of P*l*GF-1, P*l*GF-2 or $VEGF_{121}$ are modulated by bFGF or IGF-1 when all growth factors (each at 25 ng/ml, but 10 ng/ml IGF-1) act simultaneously at equimolar concentrations. We also studied the potential normalization of the barrier disturbed with combinations of growth factors by addition of the VEGF-specific Fab fragment ranibizumab or the recombinant protein aflibercept which binds VEGF and P*l*GF. Whereas 1 ng/ml VEGF-E were sufficient to impair the iBREC barrier, a higher concentration of 100 ng/ml $VEGF_{121}$ was needed to reduce TER and expression of claudin-1 over 2 days. By P*l*GF-1 or P*l*GF-2, the barrier was not affected even at the highest concentration tested (100 ng/ml) and these factors also did not modulate the effect of $VEGF_{165}$. The weak barrier derangement caused by $VEGF_{121}$ was slightly enhanced by bFGF and IGF-1. After induction of the barrier breakdown with various combinations of all growth factors included in the study, normal TER and claudin-1 expression was re-established by ranibizumab. Both VEGF inhibitors ranibizumab and aflibercept similarly reinstated lost claudin-1, even when applied at a small fraction of the clinically relevant concentrations. These results show that VEGF-A, but not P*l*GF impairs the barrier function of iBREC and that the longer isoform $VEGF_{165}$ is more potent than $VEGF_{121}$. To induce barrier dysfunction in iBREC, activation of VEGF receptor 2 – probably in concert with neuropilin-1 – seems to be sufficient because VEGF-E and $VEGF_{165}$, but not P*l*GF-1/-2 reduced TER or claudin-1 expression.

Abbreviations: bFGF, basic fibroblast growth factor; (i)BREC, (immortalized) bovine retinal endothelial cells; DME, diabetic macular edema; DR, diabetic retinopathy; EC, endothelial cells; ECGM, endothelial cell growth medium; ECGS/H, endothelial cells growth supplement/H; FCS, fetal calf serum; IGF-1, insulin-like growth factor; KRN951, N-(2-Chloro-4-((6,7-dimethoxy-4-quinolyl)oxy)phenyl)-N′-(5-methyl-3-isoxazolyl)urea; NRP-1, neuropilin-1; P*l*GF, placental growth factor; REC, retinal endothelial cells; SRM, serum-reduced medium; TER, transendothelial resistance; TJ, tight junction; VECad, vascular endothelial cadherin; VEGF, vascular endothelial growth factor A; VEGF-E, vascular endothelial growth factor E; VEGFR1, VEGF receptor 1; VEGFR2, VEGF receptor 2; ZO-1, zona occludens-1.

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1. Introduction

Clinically manifested diabetic retinopathy (DR) is accompanied by increased proliferation, migration and permeability of retinal endothelial cells (REC) which most likely result from the deregulated expression of angiogenic growth factors. Particularly, vascular endothelial growth factor A (VEGF) (Aiello et al., 1994), placental growth factor (P*l*GF) (Khaliq et al., 1998), insulin-like growth factor (IGF-1) (Meyer-Schwickerath et al., 1993), and basic fibroblast growth factor (bFGF) (Boulton et al., 1997) are considered to play important roles because elevated levels were found in the vitreous fluid of DR patients. These growth factors are stimulators of either proliferation and/or migration as confirmed by *in vitro* studies with various types of vascular cells including REC (Castellon et al., 2002; Deissler et al., 2005, 2008, 2013; Stewart et al., 2011; Yan et al., 2001). Of the different VEGF-A isoforms, $VEGF_{165}$ substantially elevates permeability of primary or immortalized bovine REC ((i)BREC)) *in vitro* (Antonetti et al., 1999; Deissler et al., 2010; Harhaj et al., 2006). To cause such a disturbance of the iBREC barrier function, $VEGF_{165}$ has to be present at a concentration of at least 25 ng/ml (~ 1.5 nM) (Deissler et al., 2011). VEGF disturbs the barrier of REC in a time-dependent manner which can be assessed by measuring the transendothelial resistance (TER) of a confluent monolayer *in vitro*: Transient reduction of TER is induced shortly after addition of $VEGF_{165}$ in primary or immortalized BREC (Antonetti et al., 1999; Harhaj et al., 2006; Deissler et al., unpublished observation), but stable reduction of the TER lasting over several days is observed more than 6 h after addition of $VEGF_{165}$ (Deissler et al., 2010; Othman et al., 2013). Whereas early changes in VEGF-induced reduction of TER in primary and immortalized BREC can be prevented by inhibition of protein kinase C (Harhaj et al., 2006; Deissler et al., unpublished observation), loss of barrier function induced by extended treatment with $VEGF_{165}$ cannot (Deissler et al., 2010). Higher permeability of (retinal) endothelial cell (EC) layers is always associated with altered intracellular localization, expression and/or modification of tight junction (TJ)-proteins (Bazzoni, 2006; Cai et al., 2011; Deissler et al., 2008, 2010, 2011; Harhaj et al., 2006; Haurigot et al., 2009; Wisniewska-Kruk et al., 2012). Short treatment with $VEGF_{165}$ over minutes to hours can result in altered localizations of the REC-expressed TJ-proteins occludin, claudin-3, claudin-5, and ZO-1 (Cai et al., 2011; Harhaj et al., 2006; Wisniewska-Kruk et al., 2012). However, claudin-1 is found down-regulated and delocalized from the plasma membrane when damaging effects of $VEGF_{165}$ on REC barrier function over an extended time period were studied *in vitro* whereas only subtle changes were observed for the other TJ-proteins (Deissler et al., 2008, 2010, 2011).

The effect of the shorter isoform $VEGF_{121}$ on cellular permeability has not been thoroughly investigated, but previous studies showed that it is a stimulator of proliferation, but not migration of REC (Deissler et al., 2008; Stewart et al., 2011). Studies on the influence of the different P*l*GF isoforms P*l*GF-1/-2 on permeability of microvascular EC are inconclusive: Whereas permeability of microvascular EC isolated from the porcine brain is elevated after treatment with P*l*GF (Vogel et al., 2007), the opposite effect was seen in human dermal microvascular EC (Brkovic and Sirois, 2007). P*l*GF-1 and P*l*GF-2 also do not disrupt the barrier function of primary BREC, but P*l*GF-1 counteracts VEGF-induced barrier dysfunction in a time-dependent manner (Cai et al., 2011).

Over-expression of IGF-1 in the retina of mice results in an increased expression of VEGF associated with breakdown of the blood-retina barrier and loss of the TJ-protein claudin-1 (Haurigot et al., 2009). However, IGF-1 does not affect the negligibly low expression of VEGF by un-stimulated (i)BREC (Deissler et al., 2011; Simorre-Pinatel et al., 1994). Accordingly, the iBREC barrier is not disturbed by IGF-1 in experiments covering a wide range of concentrations around physiological conditions, but when applied together with bFGF, an intensified VEGF-induced dysfunction was observed. This effect is most distinct when the three growth factors are present in equimolar concentrations of about 1.5 nM (Deissler et al., 2011).

The members of the VEGF-family initiate intracellular signaling through binding and activating the different VEGF receptors VEGFR1, VEGFR2 or neuropilin 1 (NRP-1), all expressed in REC (Cai et al., 2011; Deissler et al., 2011). $VEGF_{165}$ as well as $VEGF_{121}$ activate VEGFR1 and VEGFR2 (Ferrara, 2004). NRP-1 is a potent receptor for $VEGF_{165}$, but is only weakly activated by $VEGF_{121}$ (Oh et al., 2002; Pan et al., 2007). Both P*l*GF-isoforms bind to VEGFR1 and P*l*GF-2 also activates NRP-1 (Migdal et al., 1998; Park et al., 1994; Sawano et al., 1996).

VEGF induced permeability of REC is considered causative of diabetic macular edema (DME) (Nguyen et al., 2006; Qaum et al., 2001) that may appear at any stage of DR. This condition can be treated with the VEGF-binding antibody fragment ranibizumab (Ferrara et al., 2006; Mitchell et al., 2011) or with the recombinant protein aflibercept/VEGF-trap binding also to P*l*GF (Aiello et al., 1995; Holash et al., 2002; Do et al., 2012).

Based on these clinical observations and experimental data, we studied the effects of combinations of different growth factors ($VEGF_{121}$, P*l*GF-1, P*l*GF-2, bFGF, and IGF-1) with $VEGF_{165}$ on the barrier of iBREC. In addition we investigated whether VEGF-inhibition is sufficient to revert these effects using ranibizumab or aflibercept. This systematic approach including all candidate factors was chosen to gain a better understanding of their relative contributions to DME pathophysiology and consequences for therapies targeting VEGF.

The model of telomerase-immortalized iBREC is well established with distinct advantages over primary cells or rodent models (Deissler et al., 2005, 2008, 2010, 2011): Immortalizing primary cells by ectopic expression of human telomerase reverse transcriptase (TRT) is usually not associated with significant changes in important cellular processes, and the amount of human TRT in iBREC is similar to that of the bovine homologue expressed in early passages of primary BREC. Compared to primary cells, it is a distinct advantage of iBREC that these are free of contaminating cells of other types allowing more accurate and detailed studies. Furthermore, most relevant proteins are highly conserved between the human and bovine homologues.

2. Materials and methods

2.1. Reagents, growth factors and antibodies

Recombinant human growth factors bFGF, IGF-1, P*l*GF-1 (all *E. coli*-expressed) and $rhVEGF_{165}$ (SF21-expressed) were purchased from R&D Systems (Wiesbaden, Germany); $rhVEGF_{121}$ (SF21-expressed) from Calbiochem (Merck, Darmstadt, Germany), and P*l*GF-2 (*E. coli*-expressed) from PeproTech (Hamburg, Germany). Viral vascular endothelial growth factor-E (VEGF-E; Meyer et al., 1999) binding to VEGFR2 and NRP-1, and mouse monoclonal antibodies detecting actin were from Abcam (Cambrigde, UK). All growth factors stimulated proliferation of iBREC verifying their ability to induce signal transduction (Deissler et al., 2013). Rabbit polyclonal antibodies binding to human claudin-1 (JAY.8), claudin-3 (Z23.JM), claudin-5 (Z43.JK) or ZO-1 (Z-R1), as well as with AlexaFluor® 594 conjugated detection antibodies ($F(ab')_2$) were from Life Technologies (Karlsruhe, Germany) and horseradish peroxidase-conjugated detection antibodies from BioRad (Munich, Germany). Rabbit polyclonal antibodies binding to human and bovine VE-cadherin (VECad) were purchased from Acris (Herford,