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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,

Germany). The modified Fab fragment ranibizumab (Lucentis, 10 mg/ml) (Ferrara et al., 2006) of a humanized VEGF-binding antibody was a gift from Novartis Pharma GmbH (Nuremberg, Germany). Alflibercept (Eylea, 40 mg/ml; Do et al., 2012) was purchased from Bayer Pharma (Berlin, Germany). N-(2-Chloro-4-((6,7-dimethoxy-4-quinolyl)oxy)phenyl)-N′-(5-methyl-3-isoxazolyl)urea (KRN951), a tyrosine kinase inhibitor specific for VEGFR1 and VEGFR2 (Nakamura et al., 2006), was obtained from Calbiochem.

2.2. Cell cultivation

iBREC – telomerase-immortalized microvascular endothelial cells from bovine retina – were cultivated in Endothelial Cell Growth Medium MV (ECGM; Promocell Heidelberg, Germany) supplemented with 0.4% endothelial cells growth supplement/H (ECGS/H, 90 μg/ml Heparin), 10 ng/ml epidermal growth factor, 103 nM hydrocortisone and 5% fetal calf serum (FCS) on fibronectin-coated surfaces (BD Biosciences, Heidelberg, Germany) as previously described (Deissler et al., 2005, 2011). Expression of von Willebrand factor, VECad, TJ-proteins claudin-1, -3, -5, ZO-1 and occludin, as well as VEGF receptors VEGFR1, VEGFR2 and NRP-1 has been confirmed (Deissler et al., 2005, 2008, 2010, 2011). Although of bovine origin, iBREC can be stimulated with human recombinant growth factors (Deissler et al., 2005, 2008, 2010, 2011, 2013). Cells used in the experiments were at passages 20 to 35 counting from the stage of primary culture, for which stable expression of investigated proteins had been confirmed. Under these conditions, iBREC also formed a confluent monolayer 3 days after seeding as well as a tight barrier reflected by a stable transendothelial resistance of the monolayer of $\geq$50 Ohm $\times$ cm^2 which is in accordance with values reported for monolayers of primary BREC (Deissler et al., 2011; Tretiach et al., 2003).

2.3. Treatment of iBREC with growth factors and western blot analyses

Prior to experiments with confluent iBREC, ECGM was replaced by serum-reduced medium (SRM) containing 0.4% ECGS/H, 0.25% FCS, 1 μg/ml fibronectin and 103 nM hydrocortisone for 24 h. Cells were incubated for up to 3 d with single growth factors (1–100 ng/ml) or their combinations (each at 25 ng/ml, but 10 ng/ml IGF-1; corresponding to ~ 1.5 nM) in SRM, without exchanging the media during this incubation period, before cell extracts were prepared. To study whether P*l*GF-1 or P*l*GF-2 can counteract effects of VEGF$_{165}$, iBREC were pretreated with 50 ng/ml VEGF$_{165}$ for 6 h before P*l*GF-1 or P*l*GF-2 (50–100 ng/ml) were added. Cell extracts were prepared 42 h later. The potential of the different VEGF-binding proteins ranibizumab and aflibercept to restore barrier function was also investigated: After treating iBREC with growth factors for up to 2 d as described above, medium was exchanged with SRM containing the appropriate growth factors together with either 0.1–100 μg/ml ranibizumab (~ 2 to 2000 nM) or 0.25–25 μg/ml aflibercept (~ 2 to 200 nM) before cell extracts were prepared 24 h later. Blocking of barrier damaging by ranibizumab was also investigated in experiments in which cells were exposed to both VEGF$_{165}$ (50 ng/ml) and 1 or 10 μg/ml ranibizumab for 24 h. To investigate the potential of the specific VEGFR1/R2 inhibitor KRN951 to restore barrier function, iBREC were treated with 100 ng/ml VEGF$_{165}$ for 2 d, before medium was replaced by SRM supplemented with 100 ng/ml VEGF$_{165}$ and 0.5–1000 nM KRN951. Cell extracts were prepared 24 h later. Cell extracts were prepared and subsequently analyzed by Western blot as described (Deissler et al., 2011). To verify equal loading of protein, expression of actin was assessed in parallel.

2.4. Transendothelial electrical resistance of cell layers

Transendothelial electrical resistances (TER) of confluent iBREC monolayers were measured as described at several time points (2 h, 4 h, 6 h, 24 h, 30 h, 48 h, 52 h and 72 h) after addition of effectors (Deissler et al., 2010, 2011). To avoid temperature induced changes in TER, plates were kept on a warm plate at 37 °C during measurements (Blume et al., 2010). Normalized TER values were calculated in relation to the TER measured in serum-reduced medium just before addition of effectors.

2.5. Immunofluorescence staining

iBREC at a density of 2 $\times$ 10^4 cells/cm^2 were allowed to adhere to two-chamber slides (xWell, Sarstedt, Nuembrecht, Germany) coated with fibronectin or to fibronectin pre-coated membrane inserts (0.3 cm^2, pore size 0.4 μm; BioCoat, BD Biosciences) in ECGM. After having formed a confluent monolayer, cells were treated with a growth factor mix containing VEGF$_{165}$, VEGF$_{121}$, P*l*GF-1, P*l*GF-2, bFGF and IGF-1 (each at 25 ng/ml, but 10 ng/ml IGF-1) in SRM as described above. Cells were fixed in methanol at −20 °C for 15 min and antigens were detected by immunofluorescence staining as previously described. (Deissler et al., 2007, 2008, 2010)

2.6. General considerations and statistical analyses

Controls were treated in exactly the same way with medium lacking the effectors. All experiments were repeated several times and in each experiment data were generated from multiple replicates. The Mann–Whitney *U* test was used to compare sets of experimental data and differences resulting in *p*-values below 0.05 were considered significant. Results are presented in conventional box–whiskers diagrams, with the middle line representing the mean and the boundaries of the boxes the 75% and 25% percentiles or the mean and standard deviations are provided.

3. Results

3.1. Different effects of VEGF$_{121}$, P*l*GF-1 and P*l*GF-2 on iBREC barrier function

The barrier function of iBREC was assessed by measuring the TER of confluent cells and by Western blot-monitoring of the TJ-protein claudin-1. We focused on those effects on barrier function established in the cultures after more than 24 h because changes observed early after addition of growth factors were considered less relevant (Blume et al., 2010; Deissler et al., 2010, 2011). VEGF$_{165}$ decreases TER and claudin-1 expression within 2 days of treatment at concentrations $\geq$25 ng/ml, and it can be detected in the supernatant of the culture by Western-blot analyses even after extended incubation (Deissler et al., 2011). Although the related VEGF$_{121}$ may also contribute to barrier destabilization in REC, its role has previously not been investigated. The effects on the TER of confluent iBREC were therefore measured over 3 days after 10–100 ng/ml VEGF$_{121}$ had been added (Fig. 1A). Whereas 10 ng/ml VEGF$_{121}$ did not alter TER, concentrations of 25 and 50 ng/ml VEGF$_{121}$ weakly but significantly reduced TER within 1 or 2 days. This temporary effect was reversed during prolonged treatment (Fig. 1A). Earliest changes in TER were observed after treatment for 4 h but this effect was not significant. A stronger and for several days stable reduction of TER was observed in experiments with 100 ng/ml VEGF$_{121}$ (Fig. 1A). Compared to 25 ng/ml VEGF$_{165}$, the effect of VEGF$_{121}$ on TER was significantly weaker at this concentration (Fig. 1D).

Because of their deregulated expression in DR, we also studied whether P*l*GF-1 or P*l*GF-2 affected permeability of iBREC: Confluent

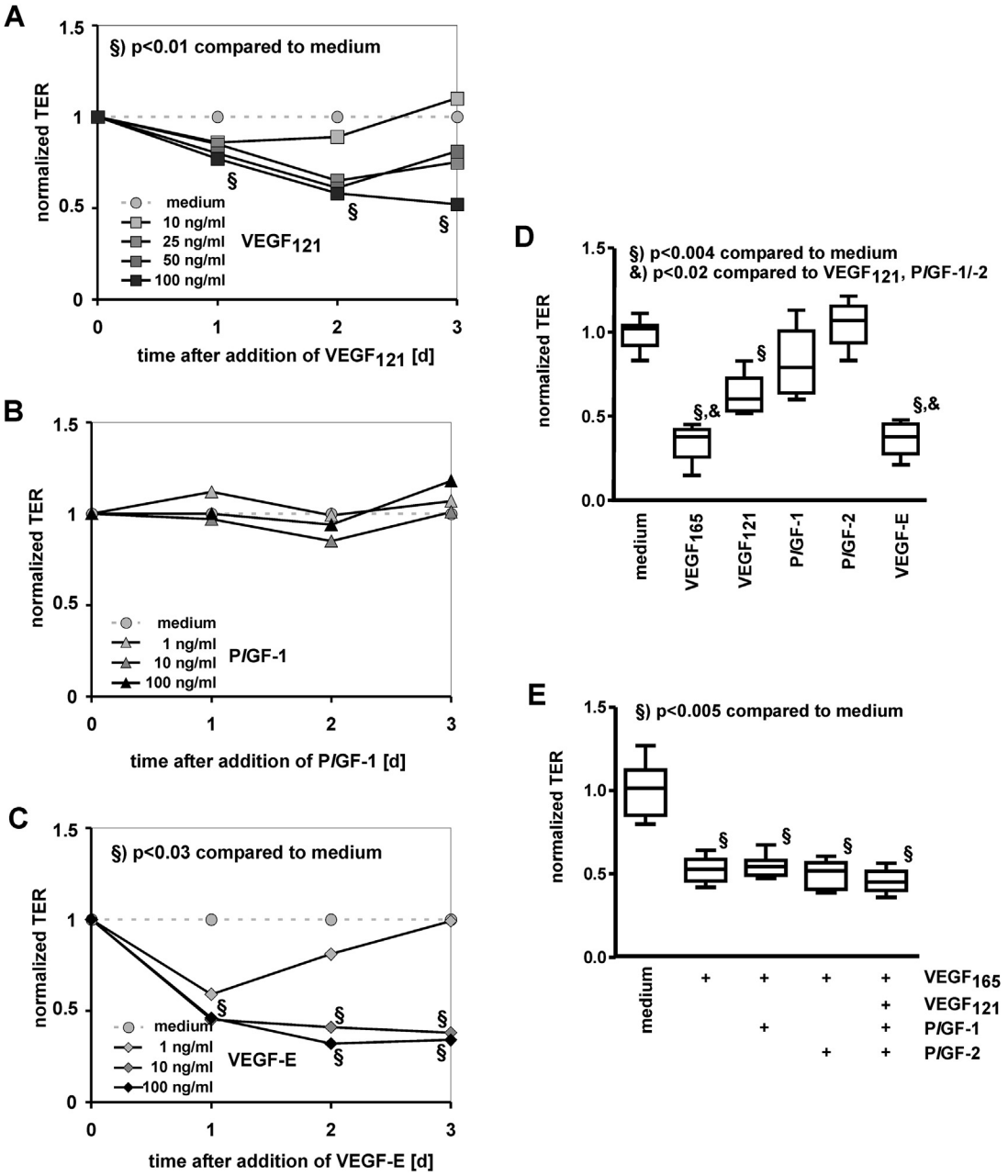

Fig. 1. Only $VEGF_{165}$ and VEGF-E strongly disturb the barrier of iBREC. **(A)**–**(C)** TER was measured at indicated time points after start of treating confluent iBREC with 1–100 ng/ml $VEGF_{121}$ **(A)**, P*l*GF-1 **(B)** or VEGF-E **(C)**. Only highest amounts of $VEGF_{121}$ resulted in a stable decrease of TER. Whereas VEGF-E efficiently reduced TER even at 1 ng/ml, P*l*GF-1 did not affect TER. Similar results obtained with P*l*GF-2 are not shown. **(D)**–**(E)** Effects of single growth factors (all applied at 25 ng/ml) **(D)** and various combinations including $VEGF_{165}$ **(E)** on TER of iBREC after 2 d of exposure. VEGF-E was as active as $VEGF_{165}$ and both were more potent than $VEGF_{121}$. P*l*GF-1 and/or P*l*GF-2 as single agents did not have any effect and also did not enhance or attenuate the effect of single $VEGF_{165}$.

cells were treated with 1–100 ng/ml P*l*GF-1, but even high amounts did not influence TER and this was also observed for P*l*GF-2 (Fig. 1B and D, data not shown). Effects induced by treatment with $VEGF_{165}$ for 2 d were not enhanced or diminished by the P*l*GFs and the combined effect of all VEGF-like factors tested was also not significantly different from that of $VEGF_{165}$ (Fig. 1E). It has been suggested that P*l*GF-1 but not P*l*GF-2 abolished $VEGF_{165}$-induced permeability of primary BREC when added 6 h after pretreatment with $VEGF_{165}$ (Cai et al., 2011). To investigate whether this effect is also present after extended exposure to $VEGF_{165}$, iBREC were pretreated with 50 ng/ml $VEGF_{165}$ for 6 h before P*l*GF-1 or P*l*GF-2 (50 and 100 ng/ml) were added. Presence of claudin-1, as marker for a functional barrier in iBREC was analyzed by Western-blot analyses in cell extracts prepared 42 h later but its loss was not counteracted by P*l*GFs (Supplementary Fig. 1A). TJ-proteins claudin-5 and ZO-1 as well as VECad were not obviously changed under either condition (Supplementary Fig. 1A). 50 ng/ml $VEGF_{165}$ significantly reduced TER after 6 h (normalized TER: 1.0 + 0.08 for medium-treated iBREC and 0.81 + 0.06 for VEGF-treated iBREC; $p = 0.0027$) and addition of either 100 ng/ml P*l*GF-1 or P*l*GF-2 6 h later also did not counteract further reduction of TER measured after 1 d (Supplementary Fig. 1B). Moreover, pretreatment with P*l*GF-1 or P*l*GF-2 did not prevent $VEGF_{165}$-induced reduction of claudin-1 or TER (data not shown). All results support the assumption that disruption of the iBREC barrier is mainly caused by $VEGF_{165}$ with only minor contributions of P*l*GFs and a limited role of the shorter VEGF-A isoform $VEGF_{121}$.

It could also be concluded that activation of VEGFR2 and/or NRP-1 might be sufficient to induce barrier dysfunction of iBREC. Therefore, the impact of the viral VEGF-E on TER was studied, because this protein activates only these VEGF receptors. VEGF-E strongly reduced TER with a threshold concentration around 10 ng/ml (Fig. 1C). Compared to 25 ng/ml $VEGF_{165}$, the effect of VEGF-E on TER was not significantly different at this concentration (Fig. 1D).

3.2. Effects of VEGFs and PlGFs in combination with bFGF and IGF-1 on iBREC barrier function

Based on our previous observation that single growth factors bFGF and IGF-1 do not change barrier properties of iBREC but slightly stretch the effect of $VEGF_{165}$ when applied in equimolar amounts (Deissler et al., 2011), we investigated whether these factors modulated the transient disturbance induced by $VEGF_{121}$.

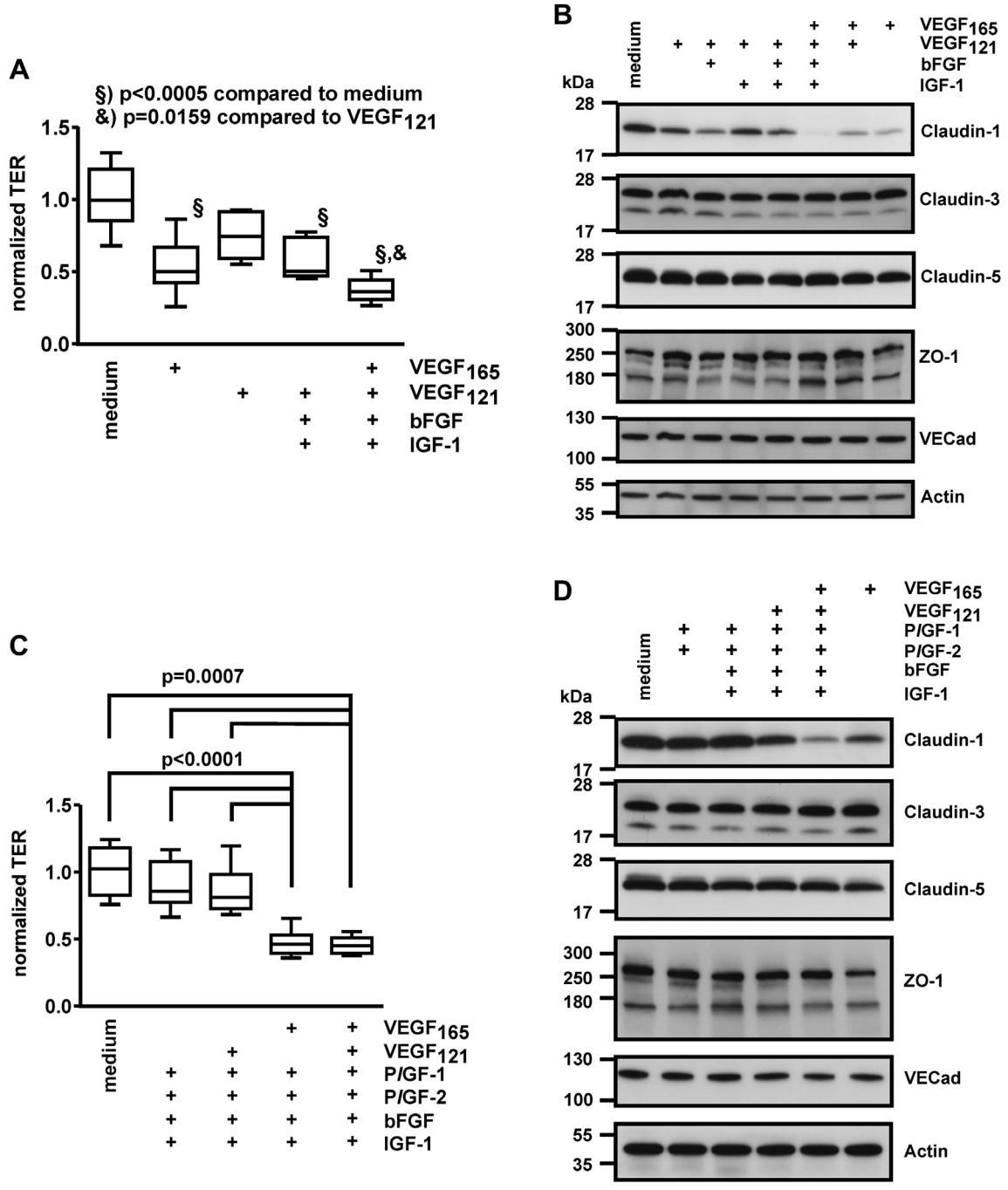

Fig. 2. Essentially $VEGF_{165}$ is responsible for changes in iBREC barrier induced by combinations of growth factors. Confluent iBREC were treated with $VEGF_{165}$, $VEGF_{121}$, bFGF and IGF-1 (each at 25 ng/ml, but 10 ng/ml IGF-1) without **(A, B)** or with **(C, D)** additional P*l*GF-1 and P*l*GF-2 (25 ng/ml each) in various combinations for 2 d. TER was measured and expression of TJ-proteins claudin-1, claudin-3, claudin-5, ZO-1 as well as VECad was analyzed by Western blot analyses. **(A)** $VEGF_{165}$ but not $VEGF_{121}$ significantly decreased TER. Addition of bFGF and IGF-1 slightly enhanced the effect of $VEGF_{121}$ which was still weaker than that of $VEGF_{165}$. **(B)** Only presence of claudin-1 but not of the other proteins tested, was strongly reduced by $VEGF_{165}$. This effect was more pronounced in the presence of bFGF and IGF-1. Supplemented with P*l*GF-1 and P*l*GF-2, bFGF and IGF-1 had no effect on TER **(C)** or claudin-1 **(D)** in the absence of $VEGF_{165}$. Expression of the other TJ-proteins and of VECad did not obviously change under these conditions confirming the importance of claudin-1 for a functional barrier.

The combination of $VEGF_{121}$ with bFGF and IGF-1 (each at 25 ng/ml, but 10 ng/ml IGF-1) slightly but significantly reduced TER whereas $VEGF_{121}$ by itself did not significantly change TER measured after treatment for 2 days (Fig. 2A). However, only further addition of 25 ng/ml $VEGF_{165}$ to this growth factor mix led to a pronounced decrease in TER (Fig. 2A). After 2 days of treatment, the effect of all four growth factors on TER treatment was not significantly different from that of single $VEGF_{165}$ (Fig. 2A). Western-blot analyses of cell extracts prepared after treatment for 2 d revealed that claudin-1 was completely lost with the combination of $VEGF_{165}$, $VEGF_{121}$, bFGF and IGF-1 and partly lost when $VEGF_{121}$ plus bFGF and IGF-1 were present (Fig. 2B). Presence of other TJ-proteins as well as VECad was not obviously changed (Fig. 2B).

The growth factor combination bFGF, IGF-1, P*l*GF-1 and P*l*GF-2 also did not significantly influence TER or claudin-1 expression (Fig. 2C and D): Significant reduction of TER was again only seen when $VEGF_{165}$ was present and its strong effect was not counteracted by P*l*GF-1 or P*l*GF-2 (Fig. 2C). Claudin-1 presence – determined by Western blot analysis – was also strongly reduced only when $VEGF_{165}$ was present in the growth factor mix (Fig. 2D). Obvious changes in the expression of other TJ-proteins and of VECad were not observed (Fig. 2D).

We have already shown that extended treatment of iBREC with 100 ng/ml $VEGF_{165}$ results in the loss of claudin-1's plasma membrane localization whereas only subtle changes for other TJ-proteins are observed (Deissler et al., 2008, 2011). Immunofluorescence staining of iBREC treated with the combination of $VEGF_{165}$, $VEGF_{121}$, bFGF, IGF-1, P*l*GF-1 and P*l*GF-2 revealed that the presence of claudin-1 is completely lost under these conditions (Fig. 3). In contrast, intense although slightly discontinuous plasma membrane staining was still observed for all the other TJ-proteins tested (claudin-3, claudin-5, ZO-1) and VECad after growth factor treatment (Fig. 3), confirming the importance of claudin-1 for a functional barrier of iBREC.

3.3. *VEGF inhibition is sufficient to restore decreases of TER and claudin-1 expression induced by $VEGF_{165}$ combined with other growth factors*

Because only $VEGF_{165}$ substantially changed barrier properties of iBREC, we assumed that its inhibition with VEGF-binding ranibizumab might re-establish a normal barrier even in the presence of other growth factors. When clinically relevant amounts of ranibizumab (100 μg/ml) were added to iBREC treated for 2 days with various combinations of $VEGF_{165/121}$, P*l*GF-1/2, bFGF and IGF-1, TER increased significantly within 24 h and reached normal values in all cases after 4 days (Fig. 4A). Ranibizumab also reinstated lost claudin-1 within 24 h in all experiments (Fig. 4B).

The possible surplus benefit of inhibiting all VEGF family members was investigated with the recombinant protein aflibercept which binds VEGF-A and P*l*GF. Reduction of TER induced by pretreating iBREC with a mix of $VEGF_{165/121}$, P*l*GF-1/2, bFGF and IGF-1 (each at 25 ng/ml, but 10 ng/ml IGF-1) was completely normalized by 25 μg/ml aflibercept (Fig. 5A). To compare their efficiencies, aflibercept or ranibizumab were added at various concentrations (2–200 nM) to the pretreated cells. Of both inhibitors even very low concentrations (≥2 nM; corresponding to ~ 0.1 μg/ml ranibizumab or ~ 0.25 μg/ml aflibercept) in comparison to those achieved in DR therapy were sufficient to reinstate lost claudin-1 completely (Fig. 5B).

3.4. *Specific inhibition of VEGF receptor tyrosine kinases is not sufficient to restore VEGF-induced barrier disturbance*

The observations that VEGF-E induced permanent changes as efficient as $VEGF_{165}$ (Fig. 1C and D) and that the effect of $VEGF_{121}$ was weaker and transient (Fig. 1A and D), suggested a crucial involvement of VEGFR2 and/or NRP-1 in barrier impairment. Accordingly, pretreatment of iBREC with the specific inhibitor KRN951 prevented VEGF-induced loss of claudin-1 at a concentration (0.5 nM) sufficient to inhibit VEGFR2 and VEGFR1 (Deissler et al., 2011; Nakamura et al., 2006). To answer the question whether inhibition of the VEGF receptor tyrosine kinases is also sufficient to *restore* already lost claudin-1, iBREC were pretreated with $VEGF_{165}$ before KRN951 (0.5 nM–1 μM) or ranibizumab (1–10 μg/ml) were added for 24 h (Fig. 6). To completely restore claudin-1, 100-times higher concentrations of KRN951 (>50 nM) were necessary than to prevent its loss (Fig. 6A), but at this concentration the inhibitor likely affects other tyrosine kinases (Nakamura et al., 2006). In contrast, preventing and re-instating loss of claudin-1 were both achieved with similar amounts of ranibizumab (Fig. 6B).

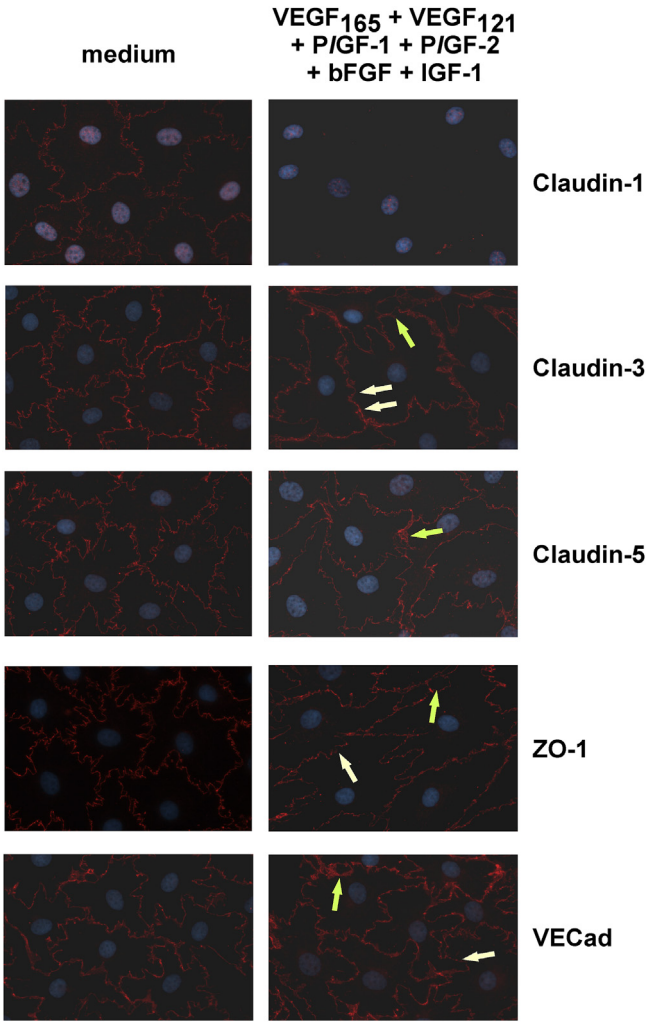

Fig. 3. Plasma membrane localization of claudin-1, but not of claudin-3, claudin-5, ZO-1 or VECad is lost after growth factor treatment. Confluent iBREC were treated with $VEGF_{165}$, $VEGF_{121}$, P*l*GF-1, P*l*GF-2, bFGF and IGF-1 (each at 25 ng/ml, but 10 ng/ml IGF-1) for 2 d before localization of claudin-1, claudin-3, claudin-5, ZO-1 and VECad was visualized by immunofluorescence staining. Plasma membrane localization of claudin-1 is lost after growth factor treatment. Slightly discontinuous staining for VECad, ZO-1 and claudin-5 is observed after growth factor treatment (yellow arrows). In addition, intracellular gaps can be detected (green arrows). Strong plasma membrane presence of all other TJ-proteins tested and VECad is seen, confirming a confluent iBREC monolayer.

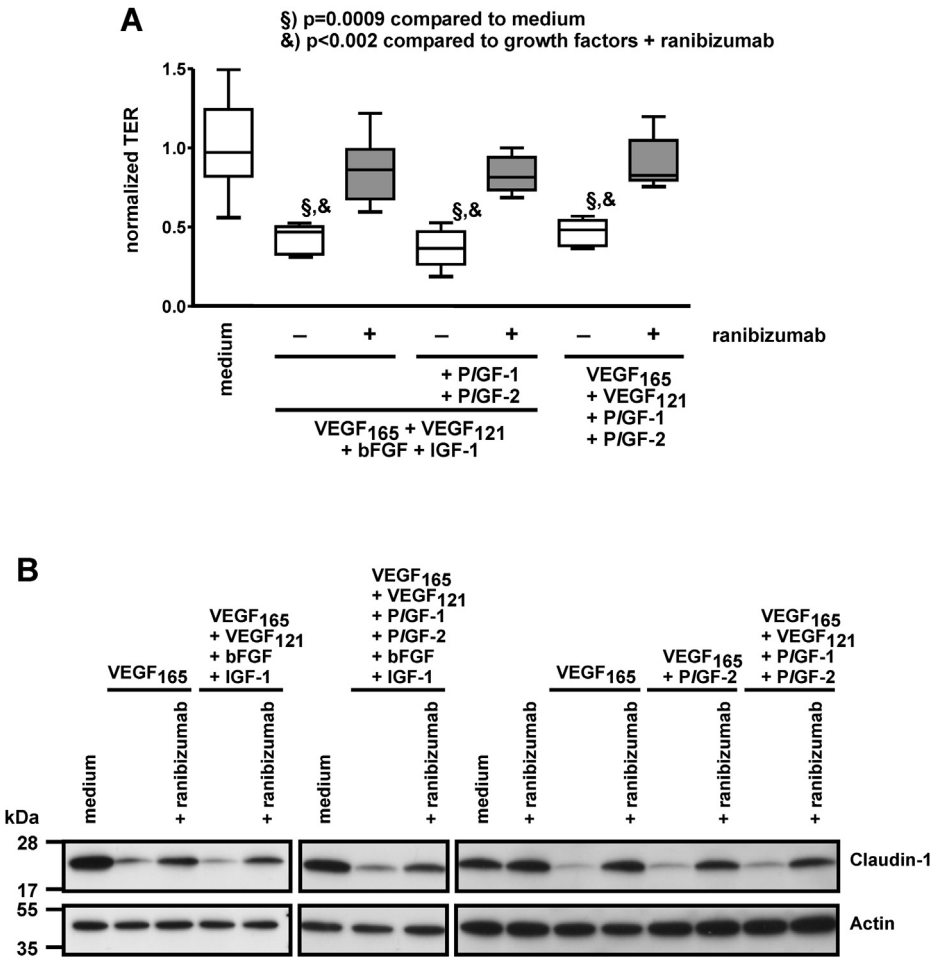

Fig. 4. TER and claudin-1 decreased by growth factor mixes are restored by inhibition of VEGF. **(A–B)**: iBREC were pretreated with various growth factor combinations (each at 25 ng/ml, but 10 ng/ml IGF-1) for 2 d before medium was changed to medium with additional 100 μg/ml ranibizumab. **(A)** TER was measured 4 days after addition of ranibizumab which then had completely restored normal TER. **(B)** Normal claudin-1 expression was also re-instated by treatment with ranibizumab for 1 d in all experimental settings.

4. Discussion

In our previous studies we showed that extended treatment of REC for up to 3 d with the isoform $VEGF_{165}$ directly results in a reduced TER associated with lost expression of claudin-1 whereas expression and cellular localization of other TJ-proteins or VECad are only subtly influenced (Deissler et al., 2008, 2010, 2011). Extending these investigations, potentially relevant effects of other members of the VEGF family were considered. Rather than studying short-term effects (Cai et al., 2011; Harhaj et al., 2006), cells were observed over several days likely corresponding to the *in vivo* situation in DR patients (Deissler et al., 2008, 2010, 2011). Growth factors and VEGF inhibitors were used at concentrations low enough to exclude non-specific effects and presence of $VEGF_{165}$ after treatment for 3 d in the culture supernatant has been confirmed (Deissler et al., 2011). As an indicator of barrier function, the TER of iBREC monolayers was determined. This indirect method is non-invasive and has the distinct advantage that the same culture can be monitored easily during long-term experiments by multiple subsequent measurements. Quantifying the flux of labeled macromolecules of different sizes through a monolayer of REC is a more direct approach to assess barrier function (Cai et al., 2011; Harhaj et al., 2006). However, the repetitive addition of dye-labeled macromolecules is associated with more frequent exchanges of the culture medium which might undesirably disturb the cultivated cells in experiments over several days. Both methods were used to study short-term (up to 1 d) exposures of REC to growth factors (Cai et al., 2011). Results of this investigation confirmed that at least for experiments of the described type, TER and flux measurements can be considered equivalent to examine barrier function.

All growth factors stimulated proliferation and/or migration of iBREC, thereby confirming that the used recombinant human polypeptides can activate their relevant bovine growth factor receptors (Deissler et al., 2005, 2008, 2013). The shorter isoform $VEGF_{121}$ only weakly and transiently disturbed the iBREC barrier function, even shortly after addition of the growth factor. Limited relevance of $VEGF_{121}$ in the regulation of permeability in endothelial cells was also shown for human dermal microvascular EC (Brkovic and Sirois, 2007) and macrovascular EC (Becker et al., 2005). In accordance with previous studies on primary BREC (Cai et al., 2011), the VEGF-related P*l*GFs did not show any direct effect on iBREC barrier. Interestingly, P*l*GF also does not induce permeability in human dermal microvascular EC (Brkovic and Sirois, 2007) but in microvascular EC of the porcine brain (Vogel et al., 2007) indicating that similar behavior even of microvascular EC of different origin is not always seen. Additive or counteracting modulations of $VEGF_{165}$'s actions by P*l*GF-1/P*l*GF-2 or $VEGF_{121}$ were also not observed in primary (Cai et al., 2011) and immortalized BREC when the growth factors were added simultaneously, suggesting that these factors play indeed a minor role in barrier impairment in REC. However, the suggested interference of

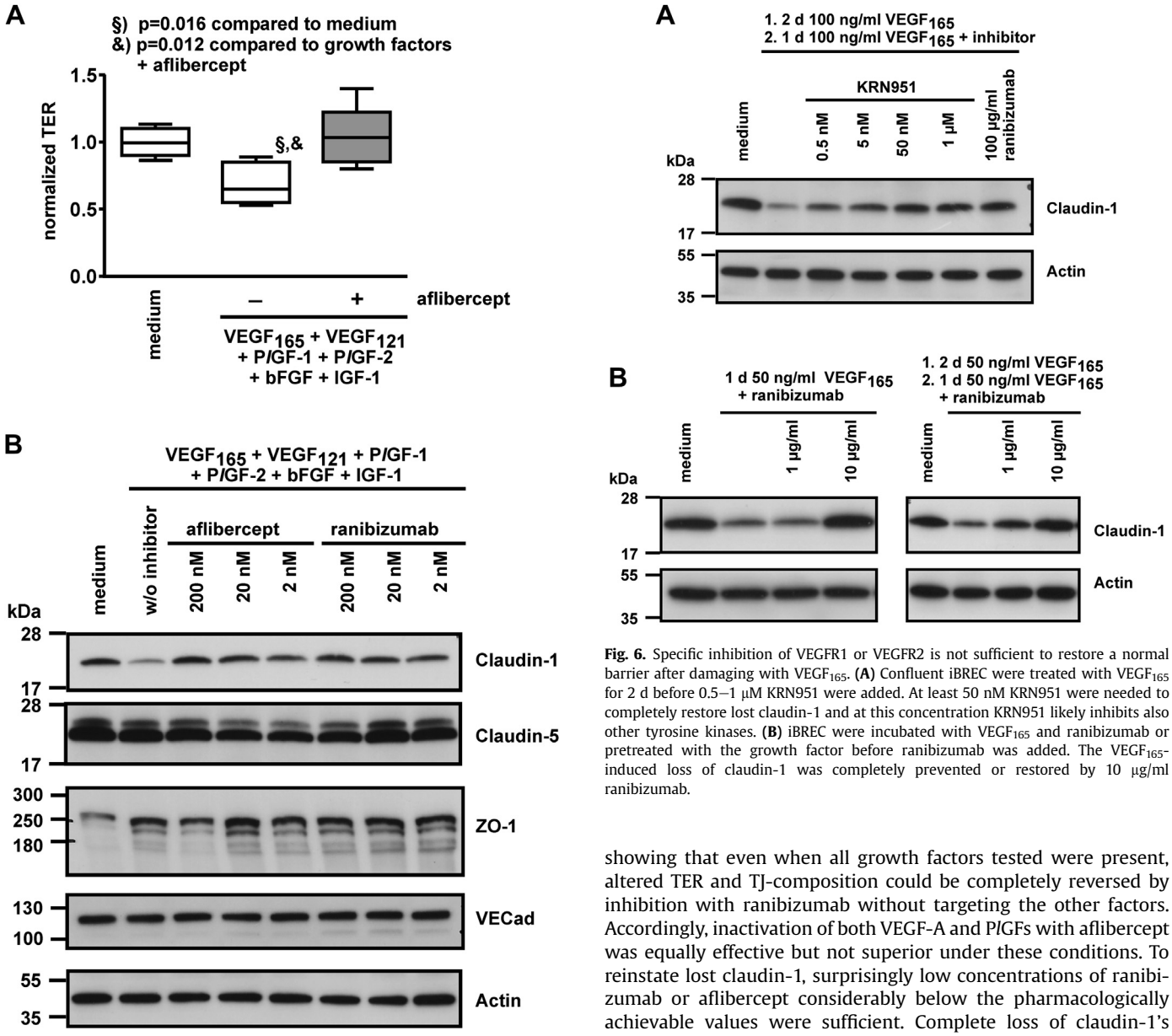

Fig. 5. Ranibizumab and aflibercept restore normal barrier function with similar efficiency. **(A)**: Confluent iBREC were treated with $VEGF_{165}$, $VEGF_{121}$, P*l*GF-1, P*l*GF-2, bFGF and IGF-1 (each at 25 ng/ml, but 10 ng/ml IGF-1) for 1 d. During further incubation with additional 25 μg/ml aflibercept for 1 d, the decreased TER was completely reverted. **(B)** iBREC were treated with growth factors and inhibitors aflibercept or ranibizumab as described above. Then claudin-1, claudin-5, ZO-1 as well as VECad were analyzed by Western blot. Both inhibitors restored lost claudin-1 with similar efficiencies. Expression of the other proteins tested was not obviously affected.

Fig. 6. Specific inhibition of VEGFR1 or VEGFR2 is not sufficient to restore a normal barrier after damaging with $VEGF_{165}$. **(A)** Confluent iBREC were treated with $VEGF_{165}$ for 2 d before 0.5–1 μM KRN951 were added. At least 50 nM KRN951 were needed to completely restore lost claudin-1 and at this concentration KRN951 likely inhibits also other tyrosine kinases. **(B)** iBREC were incubated with $VEGF_{165}$ and ranibizumab or pretreated with the growth factor before ranibizumab was added. The $VEGF_{165}$-induced loss of claudin-1 was completely prevented or restored by 10 μg/ml ranibizumab.

P*l*GF-1 with the action of $VEGF_{165}$ after delayed addition of P*l*GF-1 in primary BREC (Cai et al., 2011) was not observed in our experimental setting, most likely explained by different concentrations of vital components (e.g. hydrocortisone, FCS, heparin and fibronectin) used in the culture medium which might influence the action of $VEGF_{165}$ and/or P*l*GF on REC.

We have already shown that IGF-1 or bFGF did not alter iBREC's TER and claudin-1 expression after extended growth factor treatment (Deissler et al., 2011). Their potential contribution to an overall effect by weakly enhancing the effect of $VEGF_{121}$ also seems not relevant in the presence of the dominating factor $VEGF_{165}$. The key role of VEGF-A isoforms was strongly supported by our results showing that even when all growth factors tested were present, altered TER and TJ-composition could be completely reversed by inhibition with ranibizumab without targeting the other factors. Accordingly, inactivation of both VEGF-A and P*l*GFs with aflibercept was equally effective but not superior under these conditions. To reinstate lost claudin-1, surprisingly low concentrations of ranibizumab or aflibercept considerably below the pharmacologically achievable values were sufficient. Complete loss of claudin-1's plasma membrane localization but only subtle discontinuous staining for all other TJ-proteins tested was observed after growth factor treatment which is in accordance with previous observations after prolonged VEGF-treatment confirming claudin-1's importance for a functional iBREC barrier (Deissler et al., 2008, 2010; Wisniewska-Kruk et al., 2012). Whether loss of claudin-1 presence is associated with an altered phosphorylation pattern of the protein is unclear. However, the putative phosphorylation site Thr203 which regulates plasma membrane localization of rodent claudin-1 (Fujibe et al., 2004), is not present in the human or bovine protein which are identical in the C-terminal region of the protein.

Our results also suggest that VEGFR2 – possibly in concert with NRP-1 – is crucially involved in VEGF-induced impairment of the REC barrier: When used at physiologically relevant concentrations, $VEGF_{165}$ and VEGF-E induced a strong and permanent reduction of TER and claudin-1, the effect of $VEGF_{121}$ was weak and transient, and P*l*GF-1/-2 did not affect permeability at all. Based on these observations, we hypothesize that activation of VEGFR2 – but not VEGFR1 – is sufficient to initiate barrier disruption which is supported by experiments showing that specific inhibition of VEGFR1/2 prevents VEGF-induced loss of claudin-1 in iBREC (Deissler et al.,

2011). Activation of NRP-1 only is also insufficient, because specific inhibition of VEGF receptor tyrosine kinases prevents VEGF-induced loss of claudin-1 in iBREC (Deissler et al., 2011). However, NRP-1 probably strengthens the action of VEGFR2 and persistence of barrier break-down very likely requires bridging between NRP-1 and VEGFR2. This conclusion is based on the facts that selective inhibition of VEGFR1/2 was barely sufficient to restore the $VEGF_{165}$-induced loss of claudin-1 and that $VEGF_{121}$ which binds weakly to NRP-1 and cannot ligate NRP-1 to VEGFR2 (Pan et al., 2007), induced only transient changes. In macrovascular EC, NRP-1 regulates VEGF-induced permeability (Becker et al., 2005) and bridging between NRP-1 and VEGFR2 appears to be necessary to stimulate migration in these cells (Pan et al., 2007). In contrast, an important and independent role of VEGFR1 in the VEGF-induction of a barrier dysfunction in iBREC seems rather unlikely because both P*l*GF-isoforms did not have any effect. Contrary to these results, an involvement of VEGFR1 has been suggested, but in these studies cells from different origin (porcine brain EC) were used (Vogel et al., 2007).

Interestingly, iBREC migration was more strongly stimulated by VEGF-A together with P*l*GF compared to VEGF-A alone, although both P*l*GF-isoforms as single agents had no effect (Deissler et al., 2013). Cross-activation of VEGFR2 by P*l*GF-activated VEGFR1 shortly after addition of the growth factor has been demonstrated in studies on angiogenesis in microvascular EC (Autiero et al., 2003). The physiologically occurring heterodimer $VEGF_{165}$/P*l*GF as well as the homodimer $VEGF_{165}$ induce heterodimerization of VEGFR1 and VEGFR2 within minutes after treatment thereby also stimulating angiogenesis EC (Autiero et al., 2003). Whether cross activation or heterodimerization of the VEGF receptors are involved in the disruption of the endothelial cell barrier after prolonged treatment remains to be shown. The inability of P*l*GF-1/-2 to enhance VEGF-induced barrier dysfunction in (i)BREC suggests that an important contribution of cross activation by P*l*GF-activated VEGFR1 is rather unlikely when “long-term” effects are concerned.

Our study revealed that aflibercept and ranibizumab are similarly efficient in restoring barrier function of REC after extended treatment with growth factor mixes containing VEGF-A, P*l*GF, bFGF and IGF-1. Interestingly, superiority of additional inhibition of P*l*GF in treatment of DME using aflibercept could not be demonstrated in clinical investigations (Do et al., 2012). Taken together, a dominant role of VEGF-A in the disturbance of the blood-retina barrier observed in DME may be concluded although involvement of other factors should not be ruled out.

5. Disclosure statement, funding and role of funding source

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.exer.2013.07.018.

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