Experimental Eye Research 145 (2016) 317–326

Contents lists available at ScienceDirect

Experimental Eye Research

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

Research article

Norrin mediates angiogenic properties via the induction of insulin-like growth factor-1

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ARTICLE INFO

Article history:
Received 13 March 2015
Received in revised form
29 September 2015
Accepted in revised form 1 December 2015
Available online 17 December 2015

Keywords:
Angiogenesis
Oxygen-induced retinopathy
Retinopathy of prematurity
Norrin
β-catenin
Wnt signaling
IGF-1

ABSTRACT

Norrin is an angiogenic signaling molecule that activates canonical Wnt/β-catenin signaling, and is involved in capillary formation in retina and brain. Moreover, Norrin induces vascular repair following an oxygen-induced retinopathy (OIR), the model of retinopathy of prematurity in mice. Since insulin-like growth factor (IGF)-1 is a very potent angiogenic molecule, we investigated if IGF-1 is a downstream mediator of Norrin's angiogenic properties. In retinae of transgenic mice with an ocular overexpression of Norrin (βB1-Norrin), we found at postnatal day (P)11 a significant increase of IGF-1 mRNA compared to wild-type littermates. In addition, after treatment of cultured Müller cells or dermal microvascular endothelial cells with Norrin we observed an increase of IGF-1 and its mRNA, an effect that could be blocked with DKK-1, an inhibitor of Wnt/β-catenin signaling. When OIR was induced, the expression of IGF-1 was significantly suppressed in both transgenic βB1-Norrin mice and wild-type littermates when compared to wild-type animals that were housed in room air. Furthermore, at P13, one day after the mice had returned to normoxic conditions, IGF-1 levels were significantly higher in transgenic mice compared to wild-type littermates. Finally, after intravitreal injections of inhibitory α-IGF-1 antibodies at P12 or at P12 and P14, the Norrin-mediated vascular repair was significantly attenuated. We conclude that Norrin induces the expression of IGF-1 via an activation of the Wnt/β-catenin signaling pathway, an effect that significantly contributes to the protective effects of Norrin against an OIR.

1. Introduction

Norrin is a secreted signaling molecule that is required for the formation, differentiation and maturation of the retinal vasculature during development. In retinae of mice, the expression of Norrin is specific for Müller cells, starts after birth and last a lifetime (Ye et al., 2011). Via binding to frizzled 4 and in cooperation with its co-receptor LRP5 and TSPAN12, Norrin activates the canonical Wnt/β-catenin signaling pathway in Müller cells or microvascular endothelial cells, and hereby induces the expression of specific target genes (Xu et al., 2004; Junge et al., 2009; Ohlmann et al., 2010). In Norrin-deficient mice, a retarded development of the superficial vascular plexus and a complete lack of intraretinal capillaries was observed (Richter et al., 1998), a phenotype that was rescued in mice with an additional transgenic overexpression of Norrin in the lens (Ohlmann et al., 2005). Following an oxygen-induced retinopathy (OIR), the mouse model for retinopathy of prematurity, Norrin promoted vessel ingrowth into vaso-obliterated areas, enhanced the formation of intraretinal capillaries and blocked the formation of preretinal tufts via an activation of Wnt/β-catenin signaling (Ohlmann et al., 2010). Comparable findings were observed in mice that were treated with recombinant Norrin following an OIR (Tokunaga et al., 2013). Intriguingly, a general inhibition of Wnt signaling in retinae of *Lrp5*-deficient mice leads to a retarded development of the retinal vasculature during development and to a reduced formation of preretinal tufts following an OIR (Chen et al., 2011). Apparently, the Norrin-mediated activation of the Wnt/β-catenin signaling pathway is not only critical for the normal development of the retinal vasculature, but also for its repair after OIR.

Our knowledge about the signaling network in which the angiogenic properties of Norrin are embedded is limited. In

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http://dx.doi.org/10.1016/j.exer.2015.12.001
0014-4835/

recent work, we provided evidence that Norrin induces the expression of angiopoietin-2 which in turn contributes to Norrin's angiogenic properties on microvascular endothelial cells (Ohlmann et al., 2010). Moreover, in retinae of mice with an transgenic overexpression of Norrin in the eye, increased mRNA levels for vascular endothelial growth factor (VEGF) and placental growth factor were observed during vascular development (Ohlmann et al., 2005). Ye and coworkers demonstrated that Norrin promotes vessel formation via an induction of the transcription factor Sox17 in microvascular endothelial cells (Ye et al., 2009).

During development, the formation of the retinal vasculature depends on the expression of distinct angiogenic molecules and a tight interaction between glia cells, neurons and endothelial cells (Dorrell and Friedlander, 2006). Intriguingly, most of the angiogenic molecules that are involved in vascular development play also a role during vascular repair after injury. A prominent example is insulin-like growth factor (IGF)-1 which is involved in the development of retinal capillaries, their maintenance in the adult eye, and in vascular repair and pathology (Hellstrom et al., 2001, 2002; Shaw and Grant, 2004). In general, IGF-1 is a key mediator for body growth in childhood and has anabolic effects in adults. IGF-1 binds to the insulin-like growth factor receptor, activates phosphatidylinositide 3-kinase (PI3K)/AKT signaling, which in turn stimulates cell proliferation and growth, and inhibits apoptosis (Siddle, 2011). In particular, in cultured microvascular endothelial cells treatment with IGF-1 promotes characteristic angiogenic properties like cell proliferation, migration and tube formation (Grant et al., 1987; Shigematsu et al., 1999; Chisalita and Arnqvist, 2004). In the eye, IGF-1 and its receptors are expressed in sensory retina and retinal pigment epithelium during development and adulthood (Ocrant et al., 1989; Frade et al., 1996; Cao et al., 2001; Modanlou et al., 2006), and apparently mediate protective as well as regenerative effects on retinal neurons (Frade et al., 1996; Kermer et al., 2000; Politi et al., 2001; Rodriguez-de la Rosa et al., 2012; Dupraz et al., 2013). During development of the retinal vasculature, the expression of IGF-1 is essential (Hellstrom et al., 2001). Vice versa, the lack of IGF-1 leads to a retarded growth of retinal vessels in IGF-1-deficient mice and correlates with the presence of retinopathy of prematurity (ROP) in humans (Hellstrom et al., 2001). Along this line, the inhibition of IGF-1 signaling reduces retinal neovascularization after the induction of OIR (Smith et al., 1999; Kondo et al., 2003). In addition, increased levels for IGF-1 were found in the vitreous of patients suffering from proliferative diabetic retinopathy (Merimee et al., 1983; Grant et al., 1986), while findings in animal models strongly suggest that a raise in IGF-1 is involved in the development of diabetic retinopathy (Poulaki et al., 2004; Ruberte et al., 2004).

Quite intriguingly, Wnt/β-catenin as well as IGF-1 signaling pathways intensively crosstalk to modify each other. E.g., IGF-1 enhances Wnt/β-catenin signaling via an inhibition of the β-catenin destruction complex (Jin et al., 2008). Vice versa, β-catenin induces the expression of IGF-1 and hereby enhances its signaling (Yamauchi and Kurosaka, 2009).

Since IGF-1 appears to be a key factor for growth and maintenance of the retinal vasculature under normal and pathological conditions, and because of the intense crosstalk between Wnt/β-catenin and IGF-1 signaling, we analyzed in the present study whether Norrin might mediate angiogenic properties via IGF-1 signaling. Here we report that Norrin promotes retinal vascularization, at least in part, via the Wnt/β-catenin mediated induction of IGF-1, which in turn has the distinct potential to diminish the damaging effects of an OIR.

2. Material and methods

All animal procedures performed in this study complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and were approved by the local authorities (Regierung der Oberpfalz, Bavaria, Germany).

2.1. Oxygen-induced retinal damage

At postnatal day (P) 7, transgenic mice with an ocular overexpression of Norrin (βB1-Norrin; Ohlmann et al., 2005, 2010) and wild-type littermates, together with their nursing mothers, were exposed to 75% oxygen for 5 days (Smith et al., 1994). In order to analyze IGF-1 mRNA expression, mice were sacrificed before the onset of hyperoxia at P7, during oxygen exposure at P9, and upon or after return to room air at P12 and P13. The eyes of the mice were enucleated and dissected retinae were subjected to RNA isolation and quantitative real-time RT-PCR analyses (see below). The same procedure and the identical time pattern were applied to wild-type mice under normoxia. In addition, at P13 following oxygen-induced retinopathy, βB1-Norrin mice and wild-type littermates were sacrificed, eyes were enucleated and dissected retinae were subjected to protein isolation for Western and dot blot analyses (see below). In separate experiments, wild-type mice and transgenic littermates were intravitreally injected with 3 μl α-IGF-1 antibodies (0.2 μg/μl; R&D systems) in one eye and 3 μl 1xPBS into the fellow eye after oxygen exposure at P12 or at P12 and P14. The analysis of the effects of IGF-1 depletion on capillary regrowth into vaso-obliterated areas and on the formation of intraretinal capillaries at P14 and P17 was performed as previously described (Ohlmann et al., 2010). In brief, FITC-dextran-perfused (Sigma) retinal whole mounts were analyzed using an Axiovision fluorescent microscope with ApoTome module (Carl Zeiss). The total area of the retina, the areas of vaso-obliteration and of the deep capillary plexus were measured and calculated as area of vasobliteration per total retinal area or as area of intraretinal capillaries per total retinal area using the Axiovision software 4.8 (Carl Zeiss).

2.2. Cell culture

Müller cells were isolated from P8-P12 Wistar rat eyes as described previously (Seitz et al., 2010). The cells were cultured in DMEM containing 10% FBS, gentamicin (20 μg/ml), penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37 °C in humified 5% CO_2.

Human dermal microvascular endothelial cells (HDMEC) were purchased from Promocell (Promocell, Heidelberg, Germany) and cultured in supplemented Microvascular Endothelial Cell Growth Medium (Promocell) containing penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37 °C in humified 5% CO_2. For all experimental approaches, cell culture medium without supplement was used. Confluent HDMEC or rat Müller cells were incubated for 24 h with recombinant human Norrin (20 ng/ml and 40 ng/ml; purified as described previously Ohlmann et al., 2010) and/or DKK-1 (100 ng/ml; R&D Systems) in unsupplemented cell culture medium and subjected to RNA isolation (see below). Since Norrin and DKK-1 is highly conserved between mouse, rat and human (Ohlmann and Tamm, 2012) all experiments were performed with human recombinant Norrin and DKK-1. In addition, conditioned cell culture medium of confluent, unsupplemented HDMEC or Müller cells, treated for 36 h with Norrin (20 ng/ml), was used for IGF-1 ELISA (see below).