Long-term Treatment with Suberythropoietic Epo is Vaso- and Neuroprotective in Experimental Diabetic Retinopathy

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Key Words

Diabetic retinopathy • Erythropoietin • Pericyte loss • Acellular capillaries • Neurodegeneration

Abstract

Background/Aims: Diabetic retinopathy is characterized by pericyte loss and vasoregression both in the clinic and in animal models. A mild neurodegeneration with loss of ganglion cells is also described in the diabetic retina. Like VEGF-A, Epo is angioprotective and, in high doses, neuroprotective, however, without affecting vessel permeability. This study was to investigate the effect of a long-term suberythropoietic dose of Epo on vascular damage and neurodegeneration in a rat model of diabetic retinopathy. **Methods:** We administered Epo 3x256 IU/kg body weight/week to streptozotocin-diabetic Wistar rats for up to 6 months. Leukostasis was analyzed by quantitation of CD45 positive cells adherent to the retinal microvasculature. VEGF-A levels were assessed by Elisa at 3 months of treatment. Vasoregression was quantified in retinal digest preparations after 6 months of Epo treatment.

Neurodegeneration was analyzed from PAS stained retinal paraffin preparations. **Results:** Leukostasis was unaffected by treatment with Epo which significantly inhibited the loss of pericyte and the formation of acellular capillaries. Neurodegeneration in the diabetic retina was significantly reduced by Epo treatment. Increased VEGF-A levels in the diabetic retina were normalized by Epo treatment. **Conclusions:** Suberythropoietic Epo is effective to protect microvascular and neuronal damage in the experimental diabetic retina.

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Introduction

Retinal microvascular dysfunction is a major component in diabetic retinopathy (DR) resulting in functional and structural changes in the retina [1]. The loss of pericytes and acellular capillaries are the most significant lesions in the diabetic retina. Pericytes play an important role in the development and maturation of the vascular system [2] and are protective for endothelial cells under hyperglycemic conditions [3]. Pericyte loss

as a hallmark of human diabetic retinopathy is accompanied by endothelial cell degeneration leading to vasoregression [4]. The resulting ischemia leads to increased production of growth factors such as vascular endothelial growth factor (VEGF), and the ultimate development of proliferative diabetic retinopathy (PDR) [5, 6]. The mechanisms underlying pericyte loss and capillary non-perfusion are still not understood. Recent evidence indicated that chronic hyperglycemia is the major cause of vascular endothelial cell injury and the survival/repair mechanisms of microvascular cell injury are activated during early disease stages [3].

Increased leukocyte-endothelial cell adhesion and entrapment (referred to as retinal leukostasis) in retinal capillaries are associated with vascular non-perfusion and the development of diabetic retinopathy [7]. Increased numbers of leukocyte were observed in the diabetic retinal vasculature [8]. Retinal leukostasis increased within days of developing diabetes in humans and in rodents, and correlated with the increased expression of retinal intercellular adhesion molecule-1 (ICAM-1) and the counter-receptor CD18 [9]. ICAM-1- and CD18-deficient mice demonstrated significantly fewer adherent leucocytes, and reduced pericyte loss and formation of acellular capillaries, suggesting that CD18 and ICAM-1-mediated leukocyte adhesion may be involved in capillary non-perfusion, endothelial cell damage, and vascular leakage [8].

Recent findings further indicated that retinal neuronal cells are lost in the diabetic retina, paralleled by glial activation, resulting in neuroglial dysfunction and degeneration. The loss of retinal neurons, ganglion cells, and photoreceptors has been described in the diabetic retina [10-13], suggesting that neurodegeneration is an important component of the pathogenesis [14]. Moreover, the rescue of the microvasculature succeeded experimentally by virtue of nerve growth factor for which receptors are predominantly expressed in the neuroretina and in glial cells [10].

Clinical studies revealed that intravitreal VEGF concentration are elevated in patients with proliferative DR [15], suggesting that VEGF mediates ischemia-induced retinal neovascularization. VEGF has also been identified as an important vasculoprotective factor promoting endothelial cell (EC) survival [16]. Evenly important, VEGF is also critical as survival factor of retinal neurons and a neuroprotectant in various conditions such as ischemic injury [17], nerve regeneration [18], Parkinson's disease [19], and Alzheimer's disease [20]. Thus, early upregulation of VEGF in the diabetic retina

may represent the increased need for cellular survival support.

Apart from VEGF, there are other hypoxia-inducible growth factors with pro-survival functions for various cell types of the retina. Recently, erythropoietin [21] has been identified as an important factor stimulating angiogenesis independently of VEGF [22]. Therapeutic inhibition of Epo reduced experimental retinal neovascularization *in vivo*, and inhibited endothelial cell proliferation response to vitreous from patients with proliferative DR [23]. In addition, Epo exerts protection in different tissues injured by ischemia/reperfusion, including brain [24], retina [25], heart [26], liver [21], kidney [27], lung [28] and intestine [29]. From both, *in vitro* and *in vivo* studies, a neuroprotective effect of Epo has been deduced when applied at erythropoietic (high) concentrations.

Clinically, treatment with recombinant human Epo is efficient and safe in improving the management of the anemia associated with chronic kidney disease, but can also increase blood pressure in a dose-dependent manner [30]. Furthermore, Epo-mediated tissue protection requires concentrations of Epo, which may lead to serious vascular effects such as hypertension, infarction or stroke.

We have recently reported that low-dose Epo reduces oxidative stress and methylglyoxal-type AGEs, promotes the pro-survival signal by phosphorylation of AKT and prevents early pericyte dropout in the experimental diabetic retina [25]. Given these potentials of Epo we aimed at defining the efficacy of long-term suberythropoietic Epo on retinal vascular damage and neurodegeneration.

Materials and Methods

Diabetes induction and Epo treatment

The animal experiment in this study was performed in accordance with the Association for Research in Vision and Ophthalmology (ARVO) statement for the use of animals. All animal work was approved by the local ethics committee (Regierungspräsidium, Karlsruhe, Germany). Six-weeks-old male Wistar rats (Harlan Winkelmann GmbH, Borcheln, Germany) were rendered diabetic by intravenous (i.v.) injection of 30 mg/kg body weight (BW) streptozotocin (STZ, Roche Diagnostic, Mannheim, Germany). Once diabetes was established, diabetic rats were randomly assigned to Epo treated group (D+Epo, epoetin delta, (the only epoetin produced in a human cell line), Shire Pharmaceutical Contracts, Basingstoke, Hampshire, UK, n=20) at a dose of 256 IU/kg BW three times per week intraperitoneal (i.p.) and untreated diabetic groups (D, n=20). Age-matched rats, which had not received STZ served as non-diabetic controls (N, n=20). Body weight, blood glucose and

Glycated hemoglobin (HbA1c) was consecutively controlled throughout the study as described [25]. At the end of 3 and 6 months, eyes were removed from deeply anaesthetized animals and stored at -80°C.

One set of animals (N, D and D+Epo) was randomly assigned for telemetric assessment of blood pressure for 4 weeks as described previously [31].

Measurement of erythropoiesis

Blood cell count was performed using the CellDyn 3500™ hematoanalyzer (Abbott GmbH & Co. KG, Wiesbaden, Germany). 200 µL of EDTA-blood was collected at the end of 4, 12 and 24 weeks (n=20 per group). Counting of red blood cells (RBC), hematocrit (HCT) and Hemoglobin (HG)-value was measured photometrically as previously described [25].

Determination of exogenous human Epo in the rat retina

To ensure that the anti-human Epo antibody identifies epoietin delta, we tested a range of 1.25-20 IU/µL using dot blot analysis. Epo concentrations in a final volume of 1 µL was spotted onto a nitrocellulose membrane and dried at room temperature (RT) overnight and processed as previously described [32]. The antibody anti-human Epo (sc-1310, 1:100 in 5% milk in TBST, Santa Cruz Biotechnology, Heidelberg, Germany) was used to identify epoietin delta. Detection of administered epoietin delta in the rat retina was performed using the same anti-human Epo antibody (SC1310, Santa Cruz Biotechnology) by immunofluorescence in the diabetic rats treated for 3 months and controls [25].

Cellular EpoR expression analysis Mueller cells

To prepare suspensions of dissociated cells, isolated retinas of newborn Brown Norway rats were incubated for 30 min in calcium- and magnesium-free phosphate-buffered saline containing 0.4 mg/ml papain at 37 °C, followed by several washing steps with saline. After short incubation in saline supplemented with DNase I (200 IU/ml), the tissue pieces were fixed with 4% paraformaldehyde for 10 min, and then triturated by a 1-ml pipette tip, to obtain suspensions of isolated cells. The cells were blocked and permeabilized for 30 min with 5% normal goat serum, 0.5% Triton X-100, and 1.5% dimethylsulfoxide, and incubated in a mixture of primary antibodies of rabbit anti-EpoR (1:1000 in PBS, Santa Cruz Biotechnology, CA, USA) and mouse anti-glutamine synthetase (GS) (1:1000 in PBS, GS-6 clone, Chemicon, Hoffenheim, Germany) for 2hrs at room temperature. After washing in 1% bovine serum albumin, the secondary antibodies Cy3-coupled goat anti-rabbit (1:400, Jackson Immunoresearch, Suffolk, UK), and Cy2-coupled goat anti-mouse (1:400, Jackson) were applied for 1h at room temperature. Finally, most sections were rinsed, mounted, air-dried, and coverslipped with Entellan™ (Merck, Darmstadt, Germany).

Pericytes

Total RNA was isolated from primary human retinal pericytes (P-HRP) and an immortalized pericyte line (Bmi-HRP), using the High Pure RNA isolation kit (Roche, Mannheim,

Germany) [33]. Amplification was performed using the following cycling parameters: hold at 50°C, 30 min (RT step); hold at 95°C, 15 min (hot start to PCR); 30 cycles of 95°C (30 sec)/60°C (30 sec)/72°C (1 min), followed by a final hold at 72°C for 10 min. The RT-PCR products were visualized by electrophoresis in 2% agarose gels containing 1 µg/ml ethidium bromide. Primers used for h-EpoR were: 5' primer, CCA CAT CAA TGA AGT AGT GCT CC; 3' primer, CCA GGA TGA CCA CGA GGA TGA GG, which generated a 380-bp product.

Endothelial and smooth muscle cells

RNA was extracted from primary human umbilical vein endothelial and human aortic smooth muscle cells using Qiagen RNeasy kit 50 (74104, QIAGEN GmbH, Hilden, Germany). RNA was reverse transcribed with QuantiTect Reverse Transcription kit (Qiagen GmbH, Germany) and subjected to Taqman analysis using the Taqman 2xPCR master Mix (Applied Biosystems, Weiterstadt, Germany). Gene expression (EpoR: Hs00181092_m1) was analyzed by the 2^{-ΔΔCT} method using β-actin (Hs01064292_g1) as housekeeping control (n=4 per group). All primers and probes were purchased from Applied Biosystems (Weiterstadt, Germany).

Telemetric blood pressure measurement

To measure blood pressure continuously without disturbing the animal's natural behavior we used a previously reported protocol [34]. After implantation of the devices, STZ-diabetic rats were randomly administrated i.p. with Epo (3x256 IU/kg/week) for 4 weeks (n=5 per group). To rule out the influence of animal handling on blood pressure, the non-diabetic and diabetic groups were treated with the same amount of 0.9% saline solution. Blood pressure was measured continuously every 6 minutes throughout the 4 weeks period.

Retinal leukostasis

Retinal leukostasis, defined by CD45 positive cells adherent to the retinal microvasculature, was analyzed after 4 weeks of Epo treatment (n=5 per group). The eye was perfused with 4% Formaldehyde and immunofluorescence staining was performed using the antibody for CD45 (1:200 in PBS, DAKO Cytomation GmbH, Germany) and processed as previously reported protocol [25].

PCR-based assessment of inflammatory cytokines

Retinal RNA was isolated from individual retinas and homogenized in 1 ml Trizol reagent (Invitrogen, Karlsruhe, Germany) at 4°C according to the manufacturer's instructions (n=5 per group). Then, RNA was reverse transcribed with QuantiTect Reverse Transcription kit (Qiagen GmbH, Germany) and subjected to Taqman analysis using the Taqman 2xPCR master Mix (Applied Biosystems, Weiterstadt, Germany) as described previously (see above EpoR expression in HUVECs and hSMC). β-Actin: Rn 00667869_m1; IL6: Rn 00561420_m1; TNFα: Rn 01525859_g1; ICAM1: Rn 00564227_m1.

Retinal VEGF-A

VEGF-A levels were quantified by solid-phase sandwich ELISA (R&D systems, Mannheim, Germany) after 3 months of

Epo treatment. Retinas were isolated from frozen eyes and homogenized with 300 μ l PBS. The homogenates were frozen at -20°C overnight and were centrifuged at 12,000 $\times g$ at 4°C . Protein concentration was measured in the supernatants using Bradford method (Bio-Rad Laboratories GmbH, Munich, Germany). Samples and standards were incubated on plates coated with anti-VEGF-A ($n=5$ per group). Biotinylated antibody was added to mixtures of bound VEGF-A. To quantify binding of secondary antibody, streptavidin–peroxidase conjugate and substrate (tetramethylbenzidine) were added. After stopping the reaction by the addition of citric acid, the absorbance was measured at 450 nm. VEGF-A concentrations were determined from a standard curve.

Assessment of retinal vascular damage

Quantitative retinal morphometry was carried out in retinal digest preparations to evaluate the histological features of the retinal vasculature after 6 months ($n=6$ per group). Endothelial cells, pericytes and segments of acellular capillaries were quantified using the previous protocol [25].

Assessment of retinal neurodegeneration

Ocular paraffin sections of each group after 6 months treatment were stained with PAS and Meyer's hematoxylin (1:2, Merck, Darmstadt, Germany). Then PAS stained sections were used for retinal morphometry using the IM50 program on a Leica DMRBE microscope. For thickness measurement of retinal central layers (near to optic nerve), two images of each retina were taken at 50 $\times$ magnification. For cell number quantification of ganglion cell layer (GCL), inner nuclear layer [35] and outer nuclear layer (ONL), two images of central layers of each retinal section were taken at 200 $\times$ magnification using the same sections. Retinal thickness and the cell numbers were manually measured with the distance tool of the software ($n=6$ per group).

Statistical analysis

Data from all experiments are presented as mean $\pm$ SD. One way analysis of variance (ANOVA) was used for statistical analysis. A p -value of <0.05 was considered statistically significant.

Results

Epo treatment does not affect glycemia

In this study, we investigated the effects of long-term Epo treatment, on vascular and neuronal changes in the diabetic retina. Long-term Epo treatment did not affect body weight, which was significantly lower in diabetic rats compared to non-diabetic rats (non-diabetic (N) 570 ± 52 g, diabetic (D) 384 ± 36 g, Epo treated diabetic (D+Epo) 364 ± 33 g; Fig. 1 A). Blood glucose levels in the diabetic groups were significantly higher than in controls, and were not affected by Epo treatment (N 5.6 ± 1.3 mmol/L, D 33.3 ± 2.5 mmol/L, D+Epo 33.3 ± 2.0

Fig. 1. Metabolic parameters of non-diabetic (white circles (○), N), diabetic (white triangle (△), D) and diabetic rats treated with Epo (3x256 IU/kg/week, black triangle (▲), D+Epo). A The body weight of non-diabetic rats was significantly higher than that of diabetic and Epo treated diabetic groups. Epo treatment did not affect the body weight compared to the untreated diabetic rats. B After 6 months of Epo treatment, the increased blood glucose levels in diabetics was not changed by Epo treatment. C Glycated hemoglobin levels in the diabetic groups were significantly higher than in the non-diabetic group (***) ($p < 0.001$), but the increase was not altered by Epo treatment. Values are given as mean $\pm$ SD of 20 different animals for each group.

mmol/L; Fig. 1 B). Glycated hemoglobin levels were significantly increased in 6 months diabetic rats compared to controls ($p < 0.001$), but the increase was not changed by Epo treatment (N 5.6 ± 0.2 %, D 12.0 ± 2.0 %, D+Epo 13.6 ± 2.8 %; Fig. 1 C).

Epo treatment does not increase hematocrit

The effects of 6 months Epo treatment on the hematological variables hemoglobin, erythrocytes count and hematocrit were analyzed in samples of non-diabetic, diabetic and Epo treated diabetic rats. After 4 weeks of treatment, a modest, transient increase of HG (+14.0 %, Fig. 2 A), RBC (+11.3 %, Fig. 2 B) and HCT (+13.2 %, Fig. 2 C) was observed in Epo treated group compared to untreated diabetic control. These significant increases ($p < 0.01$) were completely normalized without dose adaptation and were indistinguishable to untreated diabetic and non-diabetic controls over the remaining period (Fig. 2 A, B and C).

Exogenous Epo binds to retinal glial cells

To investigate whether Epo at the administrated dose reaches the retina, we used dot-blot analysis to verify the recognition of epoetin delta by a commercial anti-human Epo antibody and retinal tissue obtained during the study from treated diabetic animals and controls. Fig. 3 A depicts a linear curve of increasing epoetin delta concentrations (1.25–20 IU/ μ L), indicating that the anti-human Epo antibody detected epoetin delta.

We used immunofluorescence to identify the cellular localization of exogenous Epo labeled by the antibody whose specificity was identified by dot blot analysis. Retinas of Epo treated diabetic animals revealed a clear glial staining pattern suggesting that Epo binds to retinal Mueller cells (Fig. 3 B). Specificity of the anti-human Epo antibody was shown by comparison with negative control, which was stained without this primary antibody. Colocalization of Epo with GFAP confirmed glial binding (data not shown).

To assess whether chronic hyperglycemia affected endogenous retinal Epo, we measured retinal Epo concentrations at the end of the study by Elisa. There was a reduction of endogenous Epo by diabetes. We also observed a partial effect of human on rat Epo protein. However, differences between groups did not reach statistical significance suggesting only a modest trend in changes (Fig. 4).

EpoR is expressed in Mueller cells, pericytes, endothelial cells and smooth muscle cells

The effect of therapeutic Epo administration is mediated by interactions with specific receptors- the EpoR [36, 37]. We investigated the expression of EpoR in cell lines including endothelial cells, pericytes, smooth muscle cells and glial cells. Fig. 5 A shows the colocalization of EpoR with the Mueller cell marker glutamine synthase,

Fig. 2. Effects of 6 months Epo treatment on hematological parameters. A Hemoglobin (HG) levels after 4 weeks of Epo treatment were increased compared to untreated non-diabetic and diabetic rats (** $p < 0.01$). B The numbers of red blood cells (RBC) were increased in Epo treated diabetic group compared to untreated non-diabetic and diabetic groups after 4 weeks treatment (** $p < 0.01$). C After 4 weeks of Epo treatment hematocrit (HCT) levels in Epo treated group showed an increase compared to untreated non-diabetic and diabetic rats (** $p < 0.01$). After 6 months the increased levels of RBC, HG and HCT were normalized to their initial values and Epo did not influence the RBC, HG and HCT values compared to untreated diabetic group. (White circles (○), N; white triangle (△), D; black triangle (▲), D+Epo, $n=20$ animals for each group).

suggesting that EpoR is expressed in the Mueller cells. The mRNA expression of EpoR was also found, both in human retinal pericytes and in an immortalized pericyte

Fig. 3. Recognition of epoetin delta by a commercial anti-human Epo antibody in the retina. A Illustration depicts a standard curve of increasing epoetin delta concentrations (1.25-20 IU/ μ L), indicating that the anti-human Epo antibody detected epoetin delta. B shows immunofluorescent staining of Epo on retinal paraffin sections of non-diabetic, diabetic and Epo treated groups (n=3 per group). Some cross-reactivity of the anti-human Epo antibody was noted for rat Epo in the ganglion cell layer of untreated animals. Retinas of Epo treated diabetic animals revealed a distinct glial pattern suggesting that Epo reached the target organ. Negative control was a no-primary antibody control to test for the specificity of a primary antibody involved. (GCL: ganglion cell layer, IPL: inner plexiform layer, INL: inner nuclear layer, OPL: plexiform layer, ONL: outer nuclear layer).

cell line (Fig. 5 B). EpoR was also expressed in HUVEC and in hSMC (Fig. 5 C). Thus, apart from the known presence of the EpoR in neurons [38] and ganglion cells [39], it appears that vascular and non-vascular cells express the receptor.

Suberythropoietic Epo does not affect blood pressure

Administration of erythropoietin has been reported to increase blood pressure in patients with impaired renal function and anemia [40], and in normotensive and hypertensive rats [41]. Therefore, we assessed blood pressure in a subgroup of animals that had been treated for 4 weeks after diabetes induction along with age-matched control rats. Diabetes reduced systolic blood pressure by 15 mmHg ($p < 0.01$, Fig. 6 A), and Epo did not affect systolic blood pressure. Similarly, hyperglycemia of 4 weeks' duration reduced diastolic blood pressure by 10 mmHg compared to non-diabetic rats ($p < 0.05$) and Epo treatment had no effect (Fig. 6 B).

Epo does not affect retinal leukostasis and pro-inflammatory cytokine production in the diabetic retina

Leukostasis is significantly increased in retinas of diabetic animals, and is possibly involved in endothelial

Fig. 4. Exogenous Epo injection partially affected the endogenous rat Epo concentration in the retina. After 6 months of Epo treatment (3×256 IU/kg body weight), rat Epo concentration was quantified by solid-phase sandwich ELISA (R&D systems, Mannheim, Germany) as previously described (n=5 per group). There was a reduction of endogenous Epo in the diabetic retina and a partial effect of human Epo on rat Epo protein, but the changes of rat Epo concentration between groups were not significant.

damage associated with vascular degeneration in early diabetic retinopathy [8, 42]. We evaluated, whether Epo affected leukostasis in diabetic rats. We stained retinal

Fig. 5. EpoR was expressed in Mueller cells, pericytes, endothelial cells and smooth muscle cells. A Double immunofluorescence staining of EpoR (green) with a Mueller cell marker-glutamine synthetase (GS, red) showed a colocalization of EpoR with GS, suggesting that EpoR is expressed in the Mueller cells. B The mRNA expression of EpoR was also found both in WT-human retinal pericytes (WT-HRP) and an immortalized pericyte cell line (Bmi-HRP) by RT-PCR. C The expression of EpoR in HUVEC and hSMC were detected by quantitative RT-PCR (TaqMan). EpoR is expressed both in endothelial cells (HUVEC) and in human smooth muscle cells (hSMC).

Fig. 6. Graphical presentation of the telemetrically measured blood pressures of non-diabetic (white circles, ○), diabetic (white triangle, △) and Epo treated diabetic rats (black triangle, ▲, 3x256 IU/kg BW) after 4 weeks of Epo treatment (n=5 animals for each group). A Illustration of systolic blood pressure levels during 24 hours. Compared to diabetic rats systolic blood pressure in non-diabetic rats was 15 mmHg higher (** p<0.01), and Epo treatment did not influence systolic blood pressure. B Representative recordings of diastolic blood pressure values during 24 hours. Both diabetic and Epo treated groups showed 10-mmHg reduction of diastolic blood pressures compared to non-diabetic rats (* p<0.05), and Epo had no effect.

whole mounts for CD45, a marker of leucocytes, and found that the numbers of leucocytes in arterioles (3.6 fold, $p < 0.01$), veins (2.4 fold, $p < 0.05$) and capillaries (2.4 fold, $p < 0.05$) were significantly increased in the diabetic retinas compared to non-diabetic rats. Epo treatment for 4 weeks did not affect increased leukostasis observed in the untreated diabetic retinas (Fig. 7 A). To investigate whether Epo treatment affected the pro-inflammatory cytokines, we evaluated the gene expression of $\text{TNF}\alpha$, IL6, and ICAM1 after 6 months of Epo treatment. Expression of $\text{TNF}\alpha$ and IL6 were not changed by either hyperglycemia or Epo treatment. Expression of ICAM1 was significantly increased in the diabetic retina

Fig. 7. Effect of Epo on retinal leukostasis and pro-inflammatory cytokine in the diabetic retina. A Quantitation of retinal whole mount preparations stained with CD45 to visualize adhering leucocytes in arterioles, veins and capillaries of non-diabetic, diabetic and Epo treated diabetic rats. After 4 weeks of Epo treatment the number of adhering leucocytes was comparable to the untreated diabetic group. Hyperglycemia increased the numbers of adhering leucocytes. Values are depicted as mean±SD of n= 5 of each group, ** p< 0.01, * p< 0.05. B The gene expression of TNF α , IL6, and ICAM1 was assessed by quantitative RT-PCR (TaqMan) after 6 months of Epo treatment. Gene expression of TNF α and IL6 were not changed by either hyperglycemia or Epo treatment. Expression of ICAM1 was significantly upregulated by hyperglycemia (***) p< 0.001), and treatment did not change the hyperglycemia-induced upregulation of ICAM1. (non-diabetic (□), diabetic (■) and Epo treated diabetic rats (▤, 3x256 IU/kg body weight, n= 5 per group).

compared to non-diabetic control (p< 0.001), but Epo treatment did not reduce the upregulation (Fig. 7 B). These data indicate that adhesion molecules and leukostasis are not affected by Epo treatment.

Epo reduces VEGF-A protein expression in the retina

VEGF-A has been identified as a survival factor for vascular endothelial cells and neurons [43]. We investigated protein expression of VEGF-A in the retina after 3 months of Epo treatment. The VEGF-A levels were markedly upregulated in the diabetic retina compared to the non-diabetic control (p< 0.01), and Epo treatment (3x256 IU/kg/week) reduced diabetes-induced VEGF-A expression by 48% (p< 0.05, Fig. 8).

Epo treatment prevents STZ-induced pericyte loss and the increased formation of acellular capillaries in the retina

To assess the morphological consequences of long-term Epo treatment, we quantified endothelial cell and pericytes numbers in retinal digest preparations (Fig. 9 A). After 6 months, an 18 % loss of pericytes was observed in the diabetic group (D 1,754±122 cell/mm² of capillary area vs. N 2133±76 cell/mm², p< 0.0001), which was partially (44 %) restored by Epo treatment (D+Epo 1,966±109 cell/mm² vs. D, p< 0.01, Fig. 9 B). Fig. 9 C depicts a ~2-fold increase in the amount of acellular capillary segments in diabetic rats (D 31±6 acellular

Fig. 8. VEGF-A protein expression was quantified in the retina after 3 months of Epo treatment. (non-diabetic (N), diabetic (D) and Epo treated group (D+Epo: 3x256 IU/kg body weight, n=5 animals for each group). In the retina, 3 months of diabetes induced a significant increase of VEGF-A level in the diabetic group compared to non-diabetic control (** p< 0.01) and 3 months of Epo treatment significantly reduced the upregulated VEGF-A level by hyperglycemia (* p< 0.05).

segments/10 areas vs. N 16.8±4 acellular segments/10 area, p< 0.001), Epo treatment significantly reduced the formation of acellular capillaries to non-diabetic control levels (D+Epo 15.8±5.7 vs. D 31±6, p< 0.001, Fig. 9 C). Long-term Epo treatment had no effect on the endothelial

Fig. 9. Epo treatment prevented the loss of pericytes and abolished the formation of acellular capillaries after 6 months of treatment. A Retinal digest preparations were used for the quantification of the number of endothelial cells and pericyte. (non-diabetic (N), diabetic (D) and Epo treated group (D+Epo: 3x256 IU/kg body weight). Arrows indicate acellular capillaries. B 6 months of diabetes induced an 18% reduction of pericytes compared to non-diabetics (**** $p < 0.0001$), which was inhibited by long-term Epo treatment (** $p < 0.01$). C After 6 months the significantly increased formation of acellular capillaries by 6 months of hyperglycemia (** $p < 0.001$) was almost completely abolished by Epo treatment (** $p < 0.001$). D After 6 months, the numbers of endothelial cells were comparable of all groups analyzed. Quantification was based on 6 different animals of each group.

cell numbers (Fig. 9 D). Double staining using the proliferation marker Ki67 and lectin indicated that Epo treatment did not induced excessive endothelial cell proliferation in the diabetic retina (Fig. 10).

Long-term treatment of Epo protects the retina from neurodegeneration

Based on the neuroprotective properties of erythropoietin [44], we analyzed neurodegeneration in diabetic retinas with and without Epo treatment for 6 months (Fig. 11). The diabetic retina showed a 17% reduction in the thickness of total retina compared to non-diabetic rats (D 286 ± 24 μm vs. N 345 ± 41 μm , $p < 0.05$), and Epo treatment significantly prevented retinal thinning (D+Epo 335 ± 35 μm vs. D, $p < 0.05$, Fig. 11 A, B). Hyperglycemia reduced the retinal thickness both in INL and ONL layers compared to non-diabetic controls (thickness of INL: D 48 ± 3 μm vs. N 59 ± 10 μm , $p < 0.05$; thickness of ONL: D 70 ± 3 μm vs. N 88 ± 7 μm , $p < 0.001$, Fig. 11 B). The reductions in thickness in INL and ONL of diabetic retinas were significantly prevented by Epo treatment after 6 months (INL: D+Epo 53 ± 5 μm vs. D, $p < 0.05$; ONL: D+Epo 85 ± 2 μm vs. D, $p < 0.001$, Fig. 11

Fig. 10. Suberythropoietic Epo did not affect endothelial cell proliferation in the diabetic retina. There was no significant difference of endothelial cell proliferation in the diabetic retina after 6 months of Epo treatment, indicating that suberythropoietic Epo has no proliferate properties.

B). Six months of diabetes resulted in a 25% reductions in thickness of GCL compared to the non-diabetic retina ($p < 0.05$), which were significantly prevented by Epo treatment ($p < 0.05$, Fig. 11 B).

Fig. 11. Long-term treatment (6 months) of Epo prevented retinal neurodegeneration. A Representative images of retinal sections from non-diabetic (N), diabetic (D) and Epo treated groups (D+Epo: 3x256 IU/kg body weight, n=6 animals for each group). B Illustration represented the quantification of retinal thickness. The diabetic retina showed significant retinal thinning in the ganglion cell layer (GCL), the inner nuclear layer [35], the outer nuclear layer (ONL) and the total retina. 6 months of Epo treatment significantly prevented the reductions in the thickness of GCL, INL, ONL and total retina. C The cell numbers of the GCL (per 500 μ m), INL (per 100 μ m) and ONL (per 50 μ m) significantly decreased after 6 months of diabetes compared to non-diabetic controls, and Epo treatment significantly prevented the cell loss in GCL, INL and ONL induced by hyperglycemia (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, $\square$ - N $\blacksquare$ - D $\square$ - D+Epo).

After 6 months, cell numbers in the GCL were significantly decreased by hyperglycemia compared to non-diabetic controls (cell numbers per 500 μ m GCL: D 14 ± 4 vs. N 23 ± 5 , $p < 0.01$, Fig. 11 C), and Epo treatment augmented the cell numbers by 43% (D+Epo 20 ± 3 vs. D, $p < 0.05$, Fig. 11 C). Cell numbers in the INL decreased in the 6 months diabetic groups (cell numbers per 100 μ m INL: D 34 ± 2 vs. N 40 ± 4 , $p < 0.01$, Fig. 11 C), and were normalized by Epo treatment (D+Epo 40 ± 4 vs. N, $p < 0.01$, Fig. 11 C). In the ONL, cell numbers in diabetic groups were less than in non-diabetic groups (cell numbers per 50 μ m ONL: D 45 ± 3 vs. N 54 ± 8 , $p < 0.05$), and 6 months of Epo treatment significantly prevented the cell loss induced by hyperglycemia (D+Epo 53 ± 7 vs. D, $p < 0.05$).

Discussion

Our data show that long-term suberythropoietic Epo protects the diabetic retina from vaso- and neurodege-

neration. The beneficial effect was unrelated to a change in diabetes-induced increased leukostasis, or with an impact on inflammatory gene expression suggesting that there is an alternative route of benefit for microvessels in the diabetic retina. Epo administered at low dose did not affect endothelial proliferation or blood pressure.

Long-term treatment with a suberythropoietic Epo dose prevents the formation of acellular capillaries in the diabetic retina. The prevention of acellular capillaries is the most important measure of all in experimental diabetic retinopathy, since vasoregression is strongly associated with the development and progression of diabetic retinopathy in humans [45, 46]. The mechanisms leading to acellular capillaries in the diabetic retina involve the direct effect of hyperglycemia-induced oxidative stress on endothelial cells, the indirect effect of pericyte loss which modulates survival functions on the endothelium, and an indirect effect via the neuroglia which is affected in the early diabetic retina. Our data on expression of the EpoR in endothelial cells confirms with multiple previous reports [47-49]. EpoR-mediated regulation of prosurvival

genes in endothelial cells largely compares with neuronal prosurvival effects of Epo mediated via the EpoR. According to our recent data, suberythropoietic Epo reduces glycooxidation and nitrosative stress in target organs of diabetic (micro-) angiopathy suggesting that this represent an important beneficial mechanism. However, several of the protective actions of Epo can only be obtained by high doses, not used in our study. This suggests that multiple other mechanisms of action were active by long-term use of Epo.

One of the beneficial effects is the preservation of retinal pericytes. The present study complies with our previous data that pericyte dropout is inhibited at 3 months, and expands the degree of effectivity to larger levels of pericyte dropout. Cells which express the EpoR shift to a prosurvival status upon Epo administration [37, 50]. We found the EpoR is expressed on pericyte cell lines, as well as on smooth muscle cells. Upon stimulation with Epo, three signaling cascades are triggered: the JAK2/Stat5 pathway, the PI3Kinase/AKT pathway, and the RAS/MAPK pathway. Their combined effect is the enhanced bioavailability of NO, and the induction of antiapoptotic genes such as BCL-2 [51]. As pericyte dropout can be the result of pericyte apoptosis [52], Epo may counterbalance this effect.

Epo may reduce pericyte dropout by yet another, indirect, mechanism, involving retinal glial cells. The inhibition of Ang-2 upregulation via reduction of oxidative stress and subsequent of methylglyoxal formation has been involved as a mechanism underlying pericyte migration [25]. Indeed, we and others found the EpoR expressed in retinal Mueller glia.

One marker of Mueller cell response to injury in diabetes is the upregulation of GFAP in retinal Mueller cells. We have previously reported that Epo treatment reduces GFAP upregulation in Mueller cells. Moreover, VEGF is abundantly expressed in retinal Mueller cells and upregulated by high glucose and hypoxia [32, 53, 54]. In a recent study by Krügel et al., Epo inhibited VEGF expression and osmotic swelling of Mueller cells through JAK and ERK dependant mechanisms [55]. Our data are in agreement with this concept, since we found a reduction of retinal VEGF protein expression in the Epo-treated diabetic groups.

It has been suggested that capillary non-perfusion and endothelial cell damage are associated with retinal leukostasis in early experimental diabetes [56]. A large body of evidence shows that leukocyte adhesion is increased in the retinal vasculature with long-term diabetes and the reduced expression of ICAM-1 or CD18

significantly inhibits the formation of acellular capillaries in experimental models of diabetic retinopathy, demonstrating that ICAM-1- and CD18-mediated leukocyte adhesion accounts for many of the lesions of diabetic retinopathy, such as the formation of acellular capillaries [8]. Our results are consistent with the fact that an increased number of leucocytes is present in arterioles, veins and capillaries in the diabetic retina. However, our data do not support a causal role for leukostasis in the pathogenesis of vasoregression, since we observed a prevention of the latter despite leukostasis remaining unaffected by Epo treatment. The reason for this observation is unknown, but not singular [57]. Expression of pro-inflammatory cytokines was not affected by diabetes in our model, except for ICAM1, and was not changed by Epo treatment. It is thus not likely that the positive effects of Epo were caused by a reduction of leukostasis or by inflammation in the incipient diabetic retina. As noted before, leukostasis may be associated with endothelial dysfunction caused by oxidative stress, but may not be sufficient to cause diabetic-like retinopathy.

Epo is neuroprotective in various experimental models of neural injury at high concentration [24, 58, 59]. Our findings demonstrate that Epo is neuroprotective at much lower dose. Neurodegeneration is recognized as a component of some retinal diseases including glaucoma and retinitis pigmentosa [60]. Epo prevented retinal ganglion cell damage in a STZ-induced diabetic rats model [11, 61, 62] and protected the retina from intensive light-induced photoreceptor apoptosis and subsequent retinal degeneration in rodents [63, 64]. Published data suggest the loss of retinal ganglion cells in diabetic patients and in almost all rat strains [65]. Recently, Epo and EpoR have been shown to play a critical role in neuronal development and neuroprotection [44]. After binding to the EpoR, Epo initiates a cascade of events via at least three different pathways, which lead to neuroprotection [66-68]: after binding to its receptor, both endo- and exogenous Epo induces the phosphorylation of Janus kinase 2 (JAK2), which in turn activates the phosphoinositide 3-kinase (PI3K)-serine-threonine kinase AKT, induces the translocation and activation of nuclear factor (NFκB) and stimulates homodimerization of signal transducer and activator of transcription 5 (STAT5), thereby initiating a number of downstream pathways [66]. However, we did not observe any change of P-STAT5 after Epo treatment (data not shown), and the expression of EpoR was also not changed by Epo treatment. The cellular mechanism of neuroprotection needs further evaluation. Preliminary

experiments using Tunel staining on retinal preparations did not reveal a decrease in apoptotic cells (H.P. Hammes and Q. Wang, unpublished observations).

Furthermore, we also observed that Epo treatment reduced the VEGF-A levels in the diabetic retina. VEGF affects neuronal growth, differentiation and survival. Low VEGF levels have been linked to the neuronal degeneration in animal models and human disease, indicating that VEGF might provide neuroprotection in the retina [69]. A reduction of retinal VEGF-A levels by Epo treatment indicated a reduced requirement of vaso- and neuroprotective survival signals, which suggest that Epo act as another neuroprotective, and vascular protective factor in diabetic retinopathy parallel to VEGF, and an indirect protective effect of preserved neuroretina by Epo may guard the diabetic retinal vasculature.

The amount and mode of Epo administration we chose was based on previous experiments demonstrating that hematocrit and RBC counts did not change. Brines et al. found that a lower dose of Epo is sufficient to stimulate erythropoiesis while higher doses are needed for endothelial and neuronal EpoR binding and protection [70]. Therefore, because of the lack of appropriate tools to demonstrate the presence of the EpoR in the rat eye, and to proof that Epo reached the target tissues, we performed the immunolocalization studies which suggest

that Epo treatment as performed is suitable to target the diabetic retina.

Taken together, this is the first experimental study showing that epoetin delta is effective to protect structural vascular and neuronal damage as important endpoints of experimental diabetic retinopathy. These data demonstrate that suberythropoietic doses of Epo over extended periods are beneficial for the diabetic retina, and suggest that the prevention of vasoregression is possible with much lower doses than previously assumed. Chronic sub-erythropoietic Epo may represent an effective therapeutic approach for diabetic complications in general, in particular in therapy for diabetic retinopathy.

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