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Sonic hedgehog improves ischemia-induced neovascularization by enhancing endothelial progenitor cell function in type 1 diabetes

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ABSTRACT

The Sonic hedgehog (Shh) pathway is downregulated in type 1 diabetes, and it has been reported that augmentation of this pathway may alleviate diabetic complications. However, the cellular mechanisms underlying these protective effects are poorly understood. Recent studies indicate that impaired function of endothelial progenitor cells (EPCs) may contribute to cardiovascular problems in diabetes. We hypothesized that impaired Shh signaling contribute to endothelial progenitor cell dysfunction and that activating the Shh signaling pathway may rescue EPC function and promote diabetic neovascularization. Adult male C57/B6 mice and streptozotocin (STZ)-induced type 1 diabetic mice were used. Gli1 and Ptc1 protein levels were reduced in EPCs from diabetic mice, indicating inhibition of the Shh signaling pathway. EPC migration, tube formation ability, and mobilization were impaired in diabetic mice compared with non-diabetic controls ($p < 0.05$ vs control), and all were improved by in vivo administration of the Shh pathway receptor agonist SAG ($p < 0.05$ vs diabetes). SAG significantly increased capillary density and blood perfusion in the ischemic hindlimbs of diabetic mice ($p < 0.05$ vs diabetes). The AKT activity was lower in EPCs from diabetic mice than those from non-diabetic controls ($p < 0.05$ vs control). This decreased AKT activity led to an increased GSK-3β activity and degradation of the Shh pathway transcription factor Gli1/Gli2. SAG significantly increased the activity of AKT in EPCs. Our data clearly demonstrate that an impaired Shh pathway mediated by the AKT/GSK-3β pathway can contribute to EPC dysfunction in diabetes and thus activating the Shh signaling pathway can restore both the number and function of EPCs and increase neovascularization in type 1 diabetic mice.

1. Introduction

Diabetic cardiovascular complications are the major causes of morbidity and mortality in patients with diabetes mellitus (Fuller et al., 1983; Head and Fuller, 1990). Vascular injury is mainly related to deregulation of glucose homeostasis and insulin/insulinprecursors production, generation of advanced glycation end-products, reduction in nitric oxide synthesis, and oxidative and reductive stress (Howangyin and Silvestre, 2014). It has been suggested that vascular endothelial cell dysfunction occurs early in the progression of diabetic vascular disease (Madonna and De Caterina, 2011; Porta et al., 1987). The lack of endothelial tissue regeneration and impaired angiogenesis contribute to the progression of diabetic vascular complications (Capellini et al., 2010; Waltenberger, 2001). Endothelial progenitor cells (EPCs) are a heterogeneous subpopulation of bone marrow (BM) mononuclear cells with the ability to terminally differentiate into mature endothelial cells (Asahara et al., 1997; Khoo et al., 2008). In response to vascular injury, EPCs mobilize and travel to the site of injury, regenerate the damaged endothelium, and participate in neovascularization (Roncalli et al., 2008). Accumulating evidences have suggested that the number of EPCs decreases and their proliferation, adhesion, and tube formation are significantly impaired in both type 1 and type 2 diabetic patients. These studies indicate that impaired EPC function may contribute to cardiovascular problems

Abbreviations: Shh, sonic hedgehog; EPCs, endothelial progenitor cells; STZ, streptozocin; Hh, hedgehog; BM, bone marrow.

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in diabetes (Fadini et al., 2005; Loomans et al., 2004; Tepper et al., 2002). However, the mechanisms underlying the reduced numbers of EPCs and their dysfunction are poorly understood.

The hedgehog (Hh) signaling cascade serves critical functions in cell-fate specification, tissue polarity and patterning during embryogenesis as well as the maintenance of tissue homeostasis and repair after severe injuries in postnatal and adult life (Hui and Angers, 2011; Ingham and McMahon, 2001). Hh proteins are potent morphogens, mitogens, and survival factors for a variety of cell types, including pluripotent embryonic stem cells and multipotent tissue-resident adult progenitor cells (Mimeault and Batra, 2010; Tamarat et al., 2004). The hedgehog protein family comprises Indian hedgehog, Desert hedgehog, and Sonic hedgehog (Shh), which is the most widely expressed and thoroughly studied family member.

There is increasing evidence that the Shh signaling pathway is impaired in diabetic vasculopathy and that activation of this pathway can repair the diabetic tissue damage (Asai et al., 2006; Calcutt et al., 2003; Podlasek et al., 2003). Our previous research has shown that the Shh pathway is down-regulated in the skin tissue of type 1 diabetic mice and that providing the Shh protein can promote diabetic wound healing (Luo et al., 2009). We also demonstrated that the Shh signaling pathway receptor agonist SAG can significantly increase the concentration of Shh pathway proteins and ameliorate cardiac dysfunction in the myocardial infarction model of type 1 diabetic mice (Xiao et al., 2012). Nevertheless, the mechanism by which the Hh signaling pathway reduces diabetic complications is still unknown.

Mounting evidence has demonstrated that there is a functional relationship between the Hh pathway and EPCs. Recent reports have revealed that the Shh protein promotes EPC proliferation and migration and that the thrombospondin 1 pathway contributes to BM-derived angiogenic cell dysfunction in type 1 diabetes by suppressing the Shh pathway (Fu et al., 2006; Wang et al., 2013). In this study, we examined the hypothesis that impairment of the Shh pathway contributes to EPC dysfunction and that activating the Shh signaling pathway may rescue EPC dysfunction and promote diabetic neovascularization.

2. Materials and methods

2.1. Animals and induction of type 1 diabetes

Adult male C57/B6 mice were handled in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996). The protocol was approved by the Animal Care and Use Committee of Guangzhou City (approval reference number 00022960). Male C57/B6 mice (7–8 weeks of age) received intraperitoneal injections of citrate buffer (0.05 mol/L sodium citrate, pH 4.5, control) or streptozotocin (45 mg/kg, Sigma Chemical, U.S.) dissolved in sterile citrate buffer for 5 consecutive days during the first week of the study. Mice with a blood glucose level >16.7 mmol/L were considered diabetic and used for experiments after 6–7 weeks of hyperglycemia (Luo et al., 2004; Schatteman et al., 2000).

2.2. Hindlimb ischemia and in vivo therapy

Unilateral hindlimb ischemia was induced in non-diabetic and diabetic mice 7 weeks following induction of diabetes. The proximal and distal portions of the right femoral artery were ligated, while the left femoral artery was exposed but not dissected and served as a non-ischemic control. The Shh pathway receptor agonist SAG (5 mg/kg per day; Merck) or Shh pathway receptor antagonist SANT-1 (3.3 mg/kg per day; Merck) were administered intraperitoneally after femoral artery dissection, and the dose was given once a day and continued for 14 days. Hindlimb blood flow was measured before, immediately after, 7 days after, and 14 days after right femoral artery ligations, using a laser Doppler perfusion imager system (Chen et al., 2012; Gherghe et al., 2011). On day 14, EPCs were collected and cultured and their capacity was evaluated.

2.3. Flow cytometry

A FACSCalibur flow cytometer was used to assess EPC mobilization. Mouse peripheral blood was collected on day 14 after femoral artery ligations. Freshly isolated mouse peripheral blood samples (200 μL) were incubated with (APC) anti-mouse CD34 (eBioscience, U.S.) and phycoerythrin anti-mouse Flk-1 (VEGFR-2, eBioscience, U.S.) antibodies for 30 min. Isotype-specific conjugated anti-IgG served as a negative control. Red blood cells were cleared for 10 min and washed with PBS before analysis. Circulating EPCs were determined to originate from the MNC population and were gated with double positive for $CD34^+$/$Flk\text{-}1^+$ (Chen et al., 2012; Huang et al., 2012).

2.4. EPC culture and characterization

EPCs were isolated and cultured as previously described (Mallat et al., 2002; Zhao et al., 2010). BM-MNCs were isolated by flushing the tibias and femurs of mice with PBS and layering the bone marrow on a density gradient (Histopaque 1087 [Sigma Chemical Co, U.S.]) followed by centrifugation. Isolated BM-MNCs were plated on fibronectin-coated (BD) culture dishes. Cells were cultured in Endothelial Cell Basal Medium 2 (EBM-2) (Lonza, U.S.) with 5% fetal bovine serum (FBS), vascular endothelial growth factor (VEGF), fibroblast growth factor 2 (FGF-2), epidermal growth factor, insulin-like growth factor, and ascorbic acid. After 4 days in culture, non-adherent cells were removed by washing and new media was applied.

After a further 7 days in culture at 37 °C under 5% CO_2, adherent cells were stained with DiI-acLDL (1 μg/mL, Invitrogen, U.S.) and FITC-UEA-1 (1 μg/mL, Sigma), stem cell marker CD34 (eBioscience, U.S.), and endothelial lineage marker Flk-1 (eBioscience, U.S.). The cells were observed using an inverted fluorescent microscope (Nikon).

EPCs after 7 days in culture were used for following functions and pathway experiments.

2.5. EPC tube formation assay

In vitro angiogenic activity of EPCs was determined by a Matrigel (BD Biosciences) tube formation assay as previously described (Hamada et al., 2006; Wang et al., 2011). EPC suspensions (1×10^5 cells/well) were seeded on a 2, 4-well chamber coated with 320 μL Matrigel and incubated with EGM-2 containing 5% FBS for 16 h at 37 °C. Images of tube morphology were taken using an inverted microscope (Nikon), and tube lengths were measured in 5 random fields at magnification of ×40 per sample. We used Adobe Photoshop to draw tube structures, which were defined as exhibiting a length 4 times its width, and measured tube lengths by Image-Pro Plus software.

2.6. EPC migration assay

EPC migration was evaluated using a modified Boyden chamber assay (Ii et al., 2006; Wang et al., 2011). EPC suspensions (5×10^4 cells/well) were placed in the upper chamber, and 500 μL EBM-2 with 5% FBS containing human VEGF (50 ng/mL, BD) was added into the lower chamber as a chemoattractant. The chamber