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Research article

Protective effects of bestatin in the retina of streptozotocin-induced diabetic mice

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ABSTRACT

CD13/APN (aminopeptidase N) was first identified as a selective angiogenic marker expressed in tumor vasculature and is considered a target for anti-cancer therapy. CD13 was also reported to express in non-diabetic, hypoxia-induced retinal neovascularization. Whether diabetes induces upregulation of CD13 expression in the retina is unknown. We hypothesize that at an early stage of non-proliferative diabetic retinopathy (NPDR) characterized by disruption of blood-retinal barrier (BRB) permeability is related to upregulated expression of CD13 because of its known role in extracellular matrix (ECM) degradation. The purpose of this study is to evaluate the role of CD13/APN and the therapeutic efficacy of a CD13/APN inhibitor in a mouse model of streptozotocin-induced NPDR. Hyperglycemic C57Bl/6 mice 26 weeks after streptozotocin (STZ) injection were intravitreally injected with a sustained release formulation of CD13/APN inhibitor bestatin. At 15th day of post-bestatin treatment, mouse retinas were evaluated for vascular permeability by Evans blue dye extravasation assay, fluorescent angiography of retinal vascular permeability and leukostasis. Retinal protein extracts were analyzed by Western blot to determine the effects of bestatin treatment on the expression of CD13/APN related inflammatory mediators of ECM degradation and angiogenesis. Intravitreal bestatin treatment significantly inhibited retinal vascular permeability and leukostasis. This treatment also significantly inhibited retinal expression of CD13, ECM degrading proteases (heparanase and MMP9 and angiogenic molecules (HIF-1α and VEGF). Intravitreal CD13 inhibition may relate to furthering our knowledge on the protective effect of bestatin against diabetic retinal vasculature abnormalities through inhibition of retinal permeability, leukostasis, inflammatory molecules of ECM degradation and angiogenesis.

1. Introduction

Vision loss due to diabetic retinopathy (DR) is primarily due to vascular complications (Dorrell et al., 2007). The current therapeutic options for this blinding disease are inadequate because of the complex etiology and chronic progression of this disease. According to the January 2011 National Diabetes Fact Sheet, 8.3% (25.8 million) of Americans have diabetes and ~12% of diabetics will develop DR (Ribeiro et al., 2015). Based on clinical evidence of vascular changes, DR can be divided into early nonproliferative diabetic retinopathy (NPDR) and more advanced, neovascular or proliferative diabetic retinopathy (PDR) (Ribeiro et al., 2015). NPDR is also characterized by increased blood-retinal barrier permeability resulting in leaky capillaries, release of angiogenic growth factors, and development of microaneurysm. Each of these subclinical lesions can progress to the development of clinically evident late stage of PDR. The current treatment options for PDR are conventional laser photocoagulation, photodynamic therapy, intravitreal corticosteroid and anti-vascular endothelial growth factor (anti-VEGF) treatment – all of which have limitations. Laser photocoagulation causes irreversible tissue damage resulting in vision loss (Neubauer and Ulbig, 2007), photodynamic therapy results in reversal of blood vessel proliferation after stopping the treatment (Saito et al., 2014), anti-VEGF treatment causes apoptosis of photoreceptor cells (Truong et al., 2011), and corticosteroid as an

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anti-inflammatory has local and systemic complications (Comyn et al., 2013). Early identification and therapeutic intervention is expected to block progression toward more advanced stages of human PDR. CD13/APN (aminopeptidase N) is a selective epitope first identified by phage display peptide assays in the tumor vasculature and in hypoxia-induced retinal neovessels (Pasqualini et al., 2000). CD13/APN is also reported to have selective expression in angiogenic blood vessels and little or no expression in the blood vessels of normal tissues (Pasqualini et al., 2000). In a mouse model of hypoxia-induced (not diabetes-induced) retinal neovascularization that mimics human PDR, systemic intravenous injection of CD13/APN inhibitor (bestatin) blocked retinal neovascularization (Bhagwat et al., 2001). Most animal models including streptozotocin-induced rodent develop NPDR and there is no best animal model that mimics human PDR (Robinson et al., 2012). It remains unknown whether diabetes upregulates CD13 in the retina of diabetic rodents. The enzymatic activity of CD13/APN includes ECM degradation and release of angiogenic growth factor VEGF (Fujii et al., 1995). Inflammatory leukostasis precedes the release of VEGF. Leukostasis leads to local ischemia or capillary non-perfusion, which subsequently induces HIF-1α and VEGF upregulation (Lin et al., 2011). Upregulation of VEGF also increases vascular permeability or leak and PDR (Ishida et al., 2003). Aminopeptidase N (CD13) is the partial activity of a bifunctional enzyme leukotriene A4 hydrolase (LTA4H) (Orning et al., 1994). LTA4H is bifunctional either as hydrolase or aminopeptidase. The hydrolase activity of LTA4H catalyzes the conversion of leukotriene A_4 to the inflammatory leukocyte attractant leukotriene B_4 (LTB_4) (Haeggström et al., 2002). The aminopeptidase activity of LTA4H is reported to protect cell survival in ischemia-injured retina (Husain et al., 2009). Bestatin is a small molecule inhibitor of LTA4H. Bestatin being the inhibitor of both aminopeptidase and hydrolase function of LTA4H is expected to block inflammatory leukostasis and BRB permeability through downregulation inflammatory mediators of ECM degradation and angiogenesis in the diabetic retina.

2. Materials and methods

2.1. Streptozotocin (STZ)-induced diabetes

Male C57Bl/6 mice (Taconic Labs) were injected intraperitoneally (i/p) with 55 mg/kg of STZ (Sigma Aldrich, St. Louis, MO) diluted in sterile citrate buffer (0.05 M, pH 4.5) were administered daily for four consecutive days. Control mice received i/p injection of citrate buffer (0.05 M, pH 4.5) alone. At 26 weeks after injection of STZ or citrate buffer all animals were tested for blood glucose level through tail vein puncture and using AlphaTRAK2 (Abbott Diabetes Care Inc., Alameda, CA). Animals with blood glucose levels more than 350 mg/dL were considered diabetic. All experimental protocols were approved by the Animal Care and Use Committee of Xavier University of Louisiana and were conducted in accordance with the ARVO statement for the use of Animals in Ophthalmic and Vision Research.

2.2. Treatment regimen

Mice were divided in three groups. Each group comprised of five mice. Group 1 (Normal standard control/non-diabetic), group 2 (diabetic treated with controlled release formulation only as vehicle) and group 3 (diabetic treated with bestatin in controlled release formulation). Treatment started at 26 weeks after STZ induction of diabetes. Intravitreal injection of 20 μM bestatin in 5 μL of controlled release formulation as vehicle was used. Animals were sacrificed at 15th day of post-bestatin treatment.

2.3. Preparation of alginate in-situ gel for ophthalmic controlled release formulation

The alginate *in-situ* gel formulation was prepared by first dissolving 0.4 g of sodium alginate (Sigma) in 20 mL of deionized water. Next, 45 mg of sodium chloride (Sigma) was added to a 10 mL volumetric flask along with approximately 2 mL of deionized water. After dissolution of the sodium chloride, 2 μL of a 100 mM bestatin and 5 mL of the 2% alginate solution were added. The sample was then brought to the dilution of 4 μM/μL with deionized water. For intravitreal injection, each eye received 20μM/5 μL of this formulation (Mandal et al., 2012; Liu et al., 2010; Gupta et al., 2015).

2.4. Evans blue dye assay

Each mouse was anesthetized and received an i/v injection (tail vein) 45 mg/kg Evans blue dye. Two hours later, a 0.1–0.2 mL of blood sample was obtained from re-anesthetized mice and the animals were perfused via the left ventricle with PBS followed by 1% paraformaldehyde. Eyes were enucleated and retina was dissected out. Retinas were treated with dimethylformamide (Sigma Aldrich, St. Louis, MO) overnight at 78 °C and centrifuged at 12,000 g for 15 min. Supernatant retinal extracts were tested spectrophotometrically at 620 nm (blue) and 740 nm (background). Blood samples were not treated with formamide and centrifuged at 3,500 g at 25 °C for 15 min and the supernatant was diluted 1:1000 before testing. BRB breakdown was calculated using the concentration of Evans blue in the blood and retina as follows

$$\frac{\text{Retinal Evans blue concentration (mg/mL)/retinal weight (mg)}}{\text{Blood Evans blue concentration (mg/mL)} \times \text{circulation time (hours)}}$$

2.5. BSA-FITC fluorescent angiography

Flat-mount retinas were prepared from BSA-FITC injected mice to have a qualitative angiographic evidence of vascular exudation. Diffuse and patchy hyperfluorescence areas in the perivascular region were the indicators of vascular leakage. Retinal vascular permeability was measured *in vivo* by injecting BSA-FITC (Sigma Aldrich, St. Louis, MO), 100 μg/g into the tail vein of anesthetized mice. Thirty minutes later, mice were euthanized and enucleated eyes were collected and fixed in 4% paraformaldehyde for 1 h. After fixation, flat-mount retinas were isolated and viewed under