Cellular Signalling 27 (2015) 460–469

Contents lists available at ScienceDirect

Cellular Signalling

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

Hyperglycemia enhances kidney cell injury in HIVAN through down-regulation of vitamin D receptors

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ARTICLE INFO

Article history:
Received 15 July 2013
Received in revised form 28 November 2014
Accepted 19 December 2014
Available online 24 December 2014

Keywords:
Oxidative stress
DNA damage
8-OHdG
Kidney injury

ABSTRACT

In the present study, we evaluated the effect of short term hyperglycemia on renal lesions in a mouse model (Tg26) of HIV-associated nephropathy (HIVAN). Control and Tg26 mice in groups (n = 6) were administered either normal saline (FVBN or Tg) or streptozotocin (FVBN + STZ or Tg26 + STZ). After two weeks, biomarkers were collected and kidneys were harvested. FVBN + STZ and Tg26 + STZ displayed elevated serum glucose levels when compared to FVBN and Tg26 respectively. Tg26 + STZ displayed elevated ($P < 0.05$) blood urea nitrogen (BUN) levels ($P < 0.05$) and enhanced ($P < 0.01$) proteinuria when compared to Tg26. Tg26 + STZ displayed enhanced ($P < 0.001$) number of sclerotic glomeruli and microcysts vs. Tg26. Renal tissues of Tg26 displayed down regulation of vitamin D receptor (VDR) expression and enhanced Ang II production when compared to FVBN mice. Hyperglycemia exacerbated down regulation of VDR and production of Ang II in FVBN and Tg mice. Hyperglycemia increased kidney cell reactive oxygen species (ROS) production and oxidative DNA damage in both FVBN and Tg26 mice. In *in vitro* studies, HIV down regulated podocyte VDR expression and also enhanced renin angiotensin system activation. In addition, both glucose and HIV stimulated kidney cell ROS generation and DNA damage and compromised DNA repair; however, tempol (superoxide dismutase mimetic), losartan (Ang II blocker) and EB1089 (VDR agonist) provided protection against DNA damaging effects of glucose and HIV. These findings indicated that glucose activated the RAS and inflicted oxidative stress-mediated DNA damage via down regulation of kidney cell VDR expression in HIV milieu both *in vivo* and *in vitro*.

1. Introduction

HIV-associated nephropathy (HIVAN) has been considered to be the manifestation of specific genetic (Apol1), environmental (HIV infection) and host factors [1,16,25,31]. Although the presence of Apol1 gene is associated with idiopathic focal segmental glomerulosclerosis, global glomerulosclerosis (related to hypertension) not all Apol1 positive HIV-infected patients develop kidney disease [26]. Thus, it appears that there may be other contributing factors such as the activation of renin angiotensin system in the development of glomerulosclerosis [17]. Since glucose has been reported to activate the renin angiotensin system in kidney cells [11], we asked whether it has potential to exacerbate kidney cell injury in HIVAN.

Patients with HIV infection on highly active retroviral therapy are able to maintain zero viral load [13]. However, occurrence of insulin resistance and the development of clinical diabetes has been reported to increase with the use of protease inhibitors (PIs) [32,36]. Experimental *in vitro* and *in vivo* studies also demonstrated that PIs induced impairment of the glucose transporter Glut 4 and of glucose sensing in ß-islet cells [19,33]. In clinical studies, a 7% incidence of new-onset diabetes was found in AIDS patients when oral glucose tolerance test was used [4]; however, only 3.3% diabetes was recorded when conventional parameters were used [10] In a 4-year cohort study, men receiving highly active anti-retroviral therapy (HAART) had a >4-fold increased risk of displaying diabetes when compared to HIV sero-negative men; nonetheless, discontinuation of PIs caused reversal of hyperglycemia [23]. All these studies indicate that patients with HIV infection on PIs are prone to develop hyperglycemia. Recently, Mallipattu et al., demonstrated that HIV transgene expression in kidney cells accelerated progression of diabetic nephropathy [22]. Thus, if a patient with diabetic nephropathy gets HIV infection in kidney cells the prognosis is likely to be worse. We asked if HIVAN patients developed hyperglycemia would it also accelerate the course of HIVAN? The later scenario is more likely because patients with HIV infection are living longer and

Abbreviations: BUN, Blood urea nitrogen; VDR, Vitamin D receptor; HIVAN, HIV-associated nephropathy; DN, Diabetic nephropathy; 8-OHdG, 8-hydroxydeoxyguanosine; HAART, Highly active anti-retroviral therapy; DHE, Dihydroethidium; FSGS, Focal segmental glomerulosclerosis; GGS, Global glomerulosclerosis; CGS, Collapsing glomerulosclerosis; Agt, Angiotensiongen

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http://dx.doi.org/10.1016/j.cellsig.2014.12.011
0898-6568/

use of PIs causes this population vulnerable to develop hyperglycemia [32,36].

Both glucose and HIV have been reported to activate renal cell renin angiotensin system in *in vitro* studies [5,11,30]. Interestingly, the renin angiotensin system has also been reported to play an important role in the development and progression of diabetic nephropathy (DN) as well as HIVAN, both in animal experimental models of DN and HIVAN as well as in humans [3,15,28,35]. Both inhibition of the production of Ang II and blockade of its effect, have been reported to slow down the progression of renal lesions in experimental animal models of DN and HIVAN [3,28]. Therefore, both ACE inhibitors and ARBs are being used to treat patients of DN and HIVAN. We hypothesize that the development of hyperglycemia will further activate the renin angiotensin system in HIVAN patients and will thus accelerate kidney cell injury in HIVAN.

Patients with diabetes have been demonstrated to have low vitamin D levels [24]. Emerging data suggest that majority of patients of HIV infection are also vitamin D deficient [18]. Vitamin D3 works through vitamin D receptors (VDR). Binding of VDR with vit D3 stabilizes VDR, preventing its degradation [8]. Moreover, binding of VDR with vit D3 translocates VDR to nuclear compartment, allowing it to act as a transcription factor for the required gene expression [6]. On that account, patients with low serum vitamin D levels are likely to have lower kidney cell VDR expression. Recently, we demonstrated that HIV directly down regulates kidney cell VDR expression in both *in vitro* and *in vivo* studies [5,30]. We hypothesize that high glucose levels will further down regulate kidney cell VDR expression in HIV milieu. Since liganded VDR is a negative regulator of renin [21], both glucose and HIV-induced down regulated kidney cell VDR expression will be associated with the activated renin angiotensin state. Thus, hyperglycemia by down regulating vitamin D receptors in kidney cells, may act as an adverse host factor for the progression of kidney cell injury in HIVAN.

In the present study, we have evaluated the effect of hyperglycemia on kidney cell injury in a mouse model of HIVAN. In addition, we have studied the effect of HIV and high glucose milieus on podocyte VDR expression, activation of the ROS and oxidative DNA damage.

2. Materials and methods

2.1. Tg26 mice

We have used age and sex matched FVB/N (control) and Tg26 (on FVB/N background) mice. Breeding pairs of FVBN mice were obtained from Jackson Laboratories (Bar Harbor, ME). Breeding pairs to develop Tg26 colonies were kindly gifted by Prof. Paul E. Klotman M.D., President and CEO, Baylor College of Medicine, Houston, TX). The Tg26 transgenic animal has the proviral transgene, pNL4-3: d1443, which encodes all the HIV-1 genes except *gag* and *pol* and therefore the mice are noninfectious. We are maintaining colonies of these mice in our animal facility. For genotyping, tail tips were clipped, DNA was isolated and PCR studies were carried out using following primers for Tg26.

HIV-F 5′ ACATGAGCAGTCAGTTCTGCCGCAGAC

HIV-R 3′ CAAGGACTCTGATGCGCAGGTGTG

The Ethics Review Committee for Animal Experimentation of Long Island Jewish Medical Center approved the experimental protocol.

2.2. Experimental protocol

Four weeks old age and sex matched control and Tg26 mice in groups (n = 6) were either administered normal saline (C/NS or Tg/NS) or streptozotocin (150 mg/Kg, intraperitoneal, single injection; C/STZ or Tg/STZ). After 3 days blood was collected by tail puncture to measure serum glucose levels. At the end of two weeks, the animals were anesthetized (by inhalation of isoflurane and oxygen) and sacrificed (by a massive intraperitoneal dose of sodium pentobarbital). After euthanization, blood was collected by a cardiac puncture. Both kidneys were excised; one was processed for histological and immunohistochemical studies while the other was used for RNA and protein extraction. Three-micrometer sections were prepared and stained with hematoxylin-eosin and periodic acid Schiff.

2.3. Renal disease biomarkers

Three primary phenotypes related to renal disease were characterized: blood urea, proteinuria (urinary protein/creatinine ratio), and renal histology. Proteinuria was measured by estimating urinary albumin: creatinine ratio by a kit supplied by Exocell, Philadelphia, PA.

2.4. Renal histology

Renal cortical sections were stained with hematoxylin and eosin and Periodic Acid Schiff (PAS). Renal histology was scored for both tubular and glomerular injury. Renal cortical sections were coded and examined under light microscopy. Twenty random fields (20×)/mouse were examined to score percentage of the involved glomeruli and tubules. Glomerular lesions were classified as focal segmental glomerulosclerosis (FSGS), global glomerulosclerosis (GGS), and collapsing glomerulosclerosis (CGS). A semiquantitative scale; 0 = no disease, 1 = 1–25% of tissue showing abnormalities, 2 = 26–50% and 3 = >50% of tissue affected were used for scoring the mesangial expansion. Two investigators, unaware of the experimental conditions, scored severity of renal lesions.

2.5. In vivo ROS generation by kidney cells

Frozen kidney sections (8 μM) from control and Tg26 mice were stained with the redox sensitive probe dihydroethidium (DHE). Superoxide ($O2^{-\bullet}$) is the first product of the univalent reduction of O2. DHE detects $O2^{-\bullet}$ production in mitochondria showing red fluorescence. DHE fluorescence was quantified using image J program (NIH, Bethesda, MD).

2.6. Preparation of podocytes

Human podocytes were obtained from Dr. Moin A. Saleem (Children's renal unit and academic renal unit, University of Bristol, Southmead Hospital, Bristol, UK). Human podocytes were conditionally immortalized by introducing temperature-sensitive SV40-T antigen by transfection [29]. The cells have additionally been transfected with a human telomerase construct [7]. These cells proliferate at permissive temperature (33 °C, conditionally immortalized human podocytes, CIHP) and enter growth arrest (conditionally immortalized differentiated human podocytes, CIDHP) after transfer to the non-permissive temperature (37 °C). The growth medium contains RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 1× Pen-Strep, 1 mM L-glutamine and 1× ITS (Invitrogen) to promote expression of T antigen.

3. Production of pseudotyped retroviral supernatant

Replication defective viral supernatants were prepared as published previously [12]. In brief, GFP reporter gene (from pEGFP-C1; Clontech, Palo Alto, CA) was substituted in place of *gag/pol* genes in HIV-1 proviral construct pNL4–3. This parental construct (pNL4-3:ΔG/P-GFP) was used to produce VSV.G pseudotyped viruses to provide pleiotropism and high-titer virus stocks. Infectious viral supernatants were produced by the transient transfection of 293 T cells using Effectene (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions. The HIV-1 *gag/pol* and VSV.G envelope genes were provided *in trans* using pCMV R8.91 and pMD.G plasmids, respectively (gifts of Dr. Didier Trono, Salk Institute, La Jolla, CA). As a negative control, virus was also produced from pHR-CMV-IRES2-GFP-ΔB, which contained HIV-1 LTRs and GFP