Nocturnal haemodialysis is associated with improved vascular smooth muscle cell biology

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Abstract

Background. Conventional haemodialysis (CHD) is associated with a reduction in vascular smooth muscle cells (VSMCs) proliferation and an increase in apoptosis.

Methods. VSMC proliferation, migration, apoptosis and Runx2 expression were assessed under normal conditions (n = 4) and before and after conversion from CHD to NHD (n = 15).

Results. Compared to normal, CHD is associated with a reduction in VSMC proliferation [0.49 ± 0.07 (CHD) versus 1.34 ± 0.02 RFU, P < 0.01], an augmented caspase-3 activity [0.30 ± 0.02 (CHD) versus 0.22 ± 0.02 RFU, P = 0.014] and a 1.4 ± 0.3 fold increase in Runx2 expression. After conversion to NHD, VSMC proliferation was higher than during CHD [from 0.49 ± 0.07 (CHD) to 0.68 ± 0.09 RFU, P = 0.006] and approached that of controls (1.34 ± 0.02, P > 0.05). Caspase-3 activity was restored to similar values as controls [0.26 ± 0.02 (NHD) versus 0.22 ± 0.04 (normal), P > 0.05]. Runx2 expression decreased to similar levels as normal controls. NHD enhanced dialysis dose delivery, lowered blood pressure, plasma parathyroid hormone levels and normalized plasma phosphate (from 1.7 ± 0.1 to 1.2 ± 0.1 mmol/L, P < 0.01). The reduction in plasma phosphate correlated with the change in VSMC proliferation (r = −0.71, P = 0.007).

Conclusions. We demonstrate that NHD is associated with restoration of abnormal VSMC biology in ESRD. Given the increasing importance of VSMCs in the pathogenesis of atherosclerosis and medial calcification, these data may have important implications for vascular risk in ESRD patients.

Keywords: apoptosis; nocturnal haemodialysis; phosphate; Runx2; vascular smooth muscle cell

Introduction

Atherosclerosis is accelerated in end-stage renal disease (ESRD) and contributes to the high annual mortality rate seen in conventional haemodialysis (CHD) patients [1]. Traditional cardiovascular risk factors account partially for the cardiovascular burden of our patient population. As a result, novel uraemic specific risks are being studied to explain the malignant nature of cardiovascular disease in ESRD [2].

The role of vascular smooth muscle cells (VSMCs) in the pathogenesis of atherosclerosis and medial calcification in uraemia has recently been investigated [3]. Although abnormal VSMC proliferation is linked with plaque development historically, chronic VSMC apoptosis has been associated with plaque rupture, vascular calcification and medial degeneration in subjects with and without ESRD [4]. Other investigators have examined the critical role of runt-related transcription factor 2 (Runx2) up-regulation leading to osteogenic differentiation of VSMCs in uraemia [5]. Taken together, it is reasonable to hypothesize that the abnormal ratio of VSMC proliferation/apoptosis coupled with its osteogenic transformation contributes in part to the elevated vascular risk of patients with ESRD.

Nocturnal home haemodialysis (NHD), which provides 8–10 h of renal replacement therapy during sleep, five to six nights per week, is a potentially beneficial mode of dialysis for the ESRD population. The Toronto NHD experience has documented significant cardiovascular improvements in patients following conversion (from CHD) to NHD including improved blood pressure (BP) control [6], reduction in anti-hypertensive drug requirements, improvement in endothelial dependent dilation [6], regression of left ventricular hypertrophy [7], stabilization of coronary calcification [8] and normalization of plasma phosphate levels [9]. However, a critical question that remains unanswered is whether enhanced haemodialysis improves abnormal VSMC biology in ESRD, and more specifically, if this effect is modulated to a greater extent by NHD compared to CHD.

We conducted the present study to test the following hypotheses: (1) augmentation of uraemic clearance by NHD is associated with improved VSMC biology to a greater extent than CHD; (2) the ratio of VSMC proliferation/apoptosis is directly related to biochemical parameters and uraemia control in ESRD patients; and (3) NHD is associated with a reduction in Runx2 expression compared to CHD.
Methods

This protocol was approved by the Research Ethics Board of the Toronto General Hospital, University Health Network, Toronto, Canada. Subjects included consecutive eligible patients who were converted to NHD at the University Health Network. Medically stable ESRD patients (age between 18–85 years) who had received a minimum of 3 months of CHD and were training for NHD were invited to participate in this study. All subjects were converted from CHD to NHD. None of the patients had any acute illness, hospitalization or symptomatic cardiovascular disease (including congestive heart failure, peripheral vascular disease and acute coronary syndrome). Written informed consent was obtained from each patient. Pregnant patients were excluded.

Clinical protocol

NHD patients received haemodialysis at home for 6–8 h, five to six nights per week. Vascular access was achieved through either a long-term internal jugular catheter (Uldall Catheter, Cook Critical Care, Bloomington, IN, USA) or an arteriovenous fistula. The dialysate flow rate of 350 mL per minute and the blood flow rate of 200–300 mL per minute were used. F80 polysulfone dialysers (Fresenius Medical care, Lexington, MA, USA) or Exceltra 120 dialysers (Baxter, Chicago, IL, USA) were used. CHD patients received haemodialysis for 4 h 3 times per week via similar vascular access. A blood flow rate of 400 mL per minute, a dialysate flow rate of 500–750 mL per minute and F80 polysulfone dialysers (Fresenius Medical Care, Lexington, MA, USA) were used. Unfractionated heparin was used for anticoagulation on CHD and NHD.

Dialysis dose per treatment was estimated by equilibrated Kt/V (eKt/V) as described by Daugirdas and colleagues where eKt/V = spKt/V − 0.6(spKt/V)/t + 0.03 (spKt/V = single pool Kt/V, K = delivered clearance, t = dialysis time and V = urea distribution volume) [10]. Single pool Kt/V was determined using blood urea reduction ratio [11].

Patient demographic information such as age, gender, ethnicity, aetiology of ESRD and co-morbid conditions was prospectively collected into a computerized clinical database. Clinical assessment, including weight, height, and blood pressure measurements, was performed at baseline and monthly after conversion to NHD. Prescribed cardiovascular medications were documented. These included diuretics, beta-blockers, angiotensin converting enzyme inhibitors, angiotensin receptor antagonists, digitalis, calcium channel blockers and vasodilators. Biochemical and haematological parameters (complete blood count, urea, creatinine, albumin, alkaline phosphatase, calcium, phosphate and parathyroid hormone) were obtained monthly during the same time intervals.

VSMC assays are described below. Blood samples were obtained mid-week pre-dialysis on an inter-dialytic day during CHD and 2 months after NHD (on the same day of the week). To minimize circadian variation, and replicate steady state CHD and NHD conditions, blood samples were drawn at the same time of day for all patients (a minimum of four hours after the end of a regular NHD session). In order to ascertain the appropriate normal responses, in all instances, sera from four healthy subjects were used as normal controls.

Cell culture

Primary cultures of human aortic smooth muscle cells were purchased from Cambrex (Workingham, UK). The cells were grown in D-MEM (Invitrogen, CA, USA) supplemented with 10% fetal bovine serum (Sigma Chemical Co., MO, USA) and maintained in humidified 5% CO2 at 37°C. In all experiments, prior to the cell incubation with patient serum, VSMCs at 80–90% confluence were maintained in serum-free DMEM for 48 h to render cells quiescent. VSMCs were used at the same passages (between passage 3 and 6).

VSMC proliferation

Confluent VSMCs seeded into 96-well microplates (3 × 104 cells/well) were stimulated with 5% serum (from patients and normal subjects) for 24 h and then incubated for an additional 18 h with bromodeoxyuridine (BrdU) that was added to a final concentration of 10 µM. BrdU incorporation into newly synthesized cellular DNA acting as a surrogate for cell proliferation was determined with an enzyme-linked immunosorbent assay (Roche Diagnostics, IN, USA).

VSMC migration

VSMC migration assays were performed using modified Boyden chambers with an 8-µm pore size polystyrene membrane separating the two chambers (BD Bioscience, NJ, USA). Transwell inserts were coated with human fibronectin (50 µg/mL; Sigma Chemical Co., MO, USA). VSMCs treated with 5% serum (from patients and normal subjects) were placed in the upper chamber (5 × 105 cells/chamber). Cell migration was measured after 4 h of incubation at 37°C. All non-migrant cells were removed from the upper face of the Transwell membrane with a cotton swab, and migrant cells, those attached to the lower face, were stained with Diff Quick (Dade, D¨udingen, Switzerland). VSMC migration was assessed in five random fields following staining.

VSMC apoptosis

VSMCs (2 × 106 cells/plate) were stimulated with 5% serum (from patients and normal subjects) for 24 h and then activated with tumour necrosis factor α (10 ng/mL) for 6 h. Interleukin-6 (IL-6) levels were measured in the cell culture medium with an enzyme-linked immunosorbent assay (Pierce Biotechnology, IL, USA) and Caspase-3 activities were assessed in total protein cell homogenates with a fluorometric enzyme assay (Roche Diagnostics, IN, USA). Caspase-3 activity was expressed as arbitrary units/mg protein and IL-6 concentration was expressed in pg/mL.

Runx2 expression

Total RNA was extracted from VSMCs with the RNeasy Plus Mini Kit (Qiagen, CA, USA). Reverse transcription and quantitative polymerase chain reactions were performed using Omniscript Reverse Transcriptase
addition, parathyroid hormone levels fell (from 23.6 ± 0.1, \(P\) per session) increased significantly (from 1.2 concentration was restored to normal (from 1.7 ± 0.1 mmol/L, \(P\) < 0.05) and the frequency of dialysis doubled. In addition, parathyroid hormone levels fell (from 23.6 ± 4.1 to 14.8 ± 4.4 pmol/L, \(P\) < 0.05) and plasma phosphate concentration was restored to normal (from 1.7 ± 0.1 to 1.2 ± 0.1 mmol/L, \(P\) < 0.01). Concomitantly, there was a fall in the mean arterial blood pressure from 113 ± 7 to 97 ± 1 mmHg, \(P\) < 0.05 with a decrease in vasoactive medications requirement from 2.3 to 0.9 medications per patient, \(P\) < 0.001. Specifically, seven of the study cohorts were prescribed angiotensin converting enzyme inhibitors or angiotensin receptor blockers at baseline. After 2 months of NHD, two patients remained on angiotensin converting enzyme inhibitors. Plasma albumin concentration did not change before and after conversion to NHD.

**VSMC studies**

Compared to normal, CHD was associated with a reduction in VSMC proliferation [0.49 ± 0.07 (CHD) versus 1.34 ± 0.02 RFU, \(P\) < 0.01]. Paired plasma samples obtained from the same patient while on CHD and NHD were examined to determine their ability to support VSMC proliferation. After conversion to NHD, VSMC proliferation was higher than during CHD [from 0.49 ± 0.07 (CHD) to 0.68 ± 0.09 RFU, \(P\) = 0.006] and approached that of normal control (1.34 ± 0.02, \(P\) > 0.05). The reduction in plasma phosphate correlated with the change in VSMC proliferation (\(r = -0.71, P = 0.007\)) (Figure 1).

CHD serum was associated with an augmented caspase-3 activity compared to normal [0.30 ± 0.02 (CHD) versus 0.22 ± 0.04 RFU, \(P\) = 0.014]. Caspase-3 activity was restored to similar values as normal controls [0.26 ± 0.02 (NHD) versus 0.22 ± 0.04 (normal), \(P\) > 0.05]. CHD, conventional haemodialysis; NHD, nocturnal haemodialysis.

**Results**

**Clinical observations**

Fifteen stable ESRD patients (age: 49 ± 1 years; 12 men) were studied. Of the 15 patients, there were 12 Caucasians, 1 Asian and 2 African Americans. Their ESRD was due to glomerulonephritis (\(n = 7\)), polycystic kidney disease (\(n = 3\)), diabetes (\(n = 2\)), vasculitis (\(n = 1\)), thrombotic micrangiopathy (\(n = 1\)) and hypertension (\(n = 1\)).

After 2 months of NHD, the dialysis dose received (\(Kt/V\) per session) increased significantly (from 1.2 ± 0.1 to 2.2 ± 0.1, \(P < 0.01\)) and the frequency of dialysis doubled. In addition, parathyroid hormone levels fell (from 23.6 ± 4.1 to 14.8 ± 4.4 pmol/L, \(P < 0.05\)) and plasma phosphate concentration was restored to normal (from 1.7 ± 0.1 to 1.2 ± 0.1 mmol/L, \(P < 0.01\)). Concomitantly, there was a fall in the mean arterial blood pressure from 113 ± 7 to 97 ± 1 mmHg, \(P < 0.05\) with a decrease in vasoactive medications requirement from 2.3 to 0.9 medications per patient, \(P < 0.001\). Specifically, seven of the study cohorts were prescribed angiotensin converting enzyme inhibitors or angiotensin receptor blockers at baseline. After 2 months of NHD, two patients remained on angiotensin converting enzyme inhibitors. Plasma albumin concentration did not change before and after conversion to NHD.
After conversion to NHD, IL-6 levels were similar to normal controls [5557 ± 1381 (NHD) versus 4350 ± 504 (normal) pg/mL, P = NS]. After conversion to NHD, Runx2 expression [1.01 ± 0.3 (NHD)] was similar to our reference under normal condition. CHD, conventional haemodialysis; NHD, nocturnal haemodialysis.

Discussion

Recent evidence suggests that abnormalities in VSMC contribute to atherosclerosis progression and medial calcification, which are the phenotypic sine qua non of vascular pathology in ESRD [14]. Our study demonstrated that enhanced uraemia clearance by NHD directly impacts on VSMC biology by restoring the ratio of VSMC proliferation/apoptosis. Runx2 is up-regulated under CHD condition. After conversion from CHD to NHD, Runx2 expression is similar to normal controls. In addition, there is a direct relationship between correction of plasma phosphate and the changes in VSMC proliferation. Furthermore, IL-6 levels released from VSMC is higher during CHD compared to NHD. Taken together, our data suggest that enhanced uraemia control via NHD is associated with restoration of VSMC biology.

Emerging data have confirmed the pathological consequences of VSMC apoptosis superimposed by the lack of proliferation in patients with ESRD [14]. Clarke et al. used a mouse model of inducible VSMC-specific apoptosis and demonstrated that plaque growth was increased by 2-fold and that plaque vulnerability was augmented [3,4]. Over time, VSMC apoptosis induced medial degeneration and vascular calcification. The detrimental effect of uraemia on VSMCs was demonstrated by Shroff et al. [14]. These investigators confirmed the association between medial calcification, vascular damage and VSMC apoptosis by quantifying calcium deposition and VSMC numbers in arteries from children with varying degrees of chronic kidney disease. Hydroxyapatite nanocrystals within vesicles released from damaged/dead VSMCs were demonstrated by electron microscopy suggesting that aberrant VSMCs were involved in the initiation of calcification. Although calcium deposition was already seen in pre-dialysis children, vascular damage and VSMC apoptosis were only observed in children undergoing dialysis. This important observation is consistent with the hypothesis that increased uraemia burden may induce VSMC apoptosis and damage and that CHD was not able to modify this outcome. In addition, these investigators showed that there was a paucity of total VSMC numbers within the vasculature of dialysis patients. Using Ki67 staining (as an index of cellular proliferation), arteries from dialysis patients showed a remarkable loss of VSMC proliferation (<0.5%), which would normally be induced by cell loss. In fact, the lack of VSMC proliferation has been documented consistently in other established uraemic models such as the subtotal nephrectomized rats [15]. Although the changes in phosphate related directly to the changes in VSMC proliferation, we are unable to discern the actual uraemic toxin(s) which may mediate the restoration of VSMC proliferation. Phosphate due to its intracellular storage and multi-compartmental kinetics behaves similarly to middle molecular size toxins [16]. Because of augmented frequency and duration offered by NHD, the clearance of phosphate is doubled that of CHD [9]. Similarly, other middle molecular size toxins (e.g. beta 2-microglobulin) have been shown to have higher clearances with NHD [17]. Our present data confirmed prior published literature in that uraemia increased apoptosis and reduced proliferation of normal VSMCs. In addition, intensive correction of uraemia via NHD was associated with the restoration of VSMC proliferation/apoptosis ratio. Given that the change in phosphate was related to the change in VSMC proliferation, it is tempting to speculate that retained uraemic solute(s) of middle molecular size may be responsible in part for the inappropriate VSMC proliferation seen in ESRD.

Runx proteins mediate transcriptional control of bone cell differentiation [18]. Aberrant Runx2 expression in
VSMC has been linked to osteogenic transformation and vascular calcification in uraemia [19–21]. To date, phosphorus overload and uraemic serum under CHD conditions have been consistently noted to induce Runx2 up-regulation in VSMC [22,23]. Our present results add to the body of work by demonstrating that correction uraemia burden by NHD is associated normalization of Runx2 expression. Future experiments may include the identification of putative uraemic toxin(s) leading to the aberrant osteogenic transformation of VSMC.

Sustained inflammation is associated with cardiovascular death in ESRD. Of various inflammatory markers, high IL-6 levels have consistently been associated with elevated cardiovascular event rates in dialysis patients [24]. Prior published literature had demonstrated that VSMC apoptosis led to the degeneration of the media layer and the development of the cystic necrotic core within blood vessels of ESRD patients [4]. VSMCs expressed IL-6 constitutively and could be induced via biomechanical stress especially in atherosclerotic segments of blood vessels [25]. Our cell culture data demonstrated that VSMCs under CHD condition were capable of releasing higher amounts of IL-6 in comparison to NHD condition. The clinical utility of plasma IL-6 levels as a marker of VSMC numbers and functions in ESRD patient requires further exploration.

In summary, we highlight the concept that augmented uraemia clearance using NHD is associated with restoration of several parameters of VSMC biology. Our results are limited by this study’s observational nature. Given that diabetes represents the most common cause of ESRD in the developed countries, the present patient cohort is not representative of the general ESRD population. The independent effect of diabetes with and without uraemia on VSMC biology requires additional investigation. Future experiments correlating our in vitro results with various in vivo vascular function tests are needed. The present study represents the first attempt to study the impact of intensive haemodialysis on VSMC biology. Given the important role of VSMCs in atherosclerosis progression and vascular calcification, the present observation adds support to the growing cardiovascular benefits of NHD and provides a rationale for further testing the impact of NHD on cardiovascular outcomes in ESRD patients.

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