Original Paper

The Impact of Osteocalcin, Osteoprotegerin and Osteopontin on Arterial Stiffness in Chronic Renal Failure Patients on Hemodialysis

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Key Words

Hemodialysis • Vascular stiffness • Pulse wave velocity • Osteocalcin • Osteoprotegerin • Osteopontin

Abstract

Background/Aims: This cross-sectional study was designed to assess the relationship between vascular stiffness (VS) and bone-related proteins involved in the development of arteriosclerosis in patients on regular hemodialysis (HD). **Methods:** 68 consecutive patients in stable clinical condition who received regular HD in the FMC Dialysis Center, Pécs were included. VS parameters (carotid-femoral pulse wave velocity – PWV, aortic augmentation index – AIx) were determined by applanation tonometry (SphygmoCor, AtCor Medical, Sidney) and the routine laboratory test were completed with measurements of osteocalcin (OC), osteopontin (OP) and osteoprotegerin (OPG) by using commercially available ELISA kits. 35 healthcare workers served as controls. **Results:** In patients on regular HD PWV markedly increased and there was several-fold elevation in the interrelated bone-specific proteins (OC, OP, OPG). PWV was found to be independently associated only with OC (β : -0.25, $p < 0.029$) and age ($r = 0.411$, $p < 0.000$), but risk factors for arterial calcification had significant impact on OC (systolic blood pressure, hsCRP, BMI), OPG (age, BMI) and OP (LDL-cholesterol). **Conclusion:** Except for OC, our results failed to document direct association of vascular lesion with OP and OPG, therefore their high circulating levels may be an epiphenomenon or they may have counter-regulatory role to attenuate the uremic calcification process.

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Introduction

The concept of accelerated arteriosclerosis has been well-established in patients with chronic renal failure (CRF) with much higher incidence of cardiovascular complications in these patients than those in the general population even after adjustment for traditional cardiovascular risk factor such as hypertension, diabetes mellitus or dyslipidemia [1, 2].

Although the underlying mechanisms of the initiation and progression of cardiovascular diseases have not been clearly defined, the contribution of non-traditional risk factors including low-grade inflammation [3], oxidative stress, endothelial dysfunction, advanced glycation end-products and dysregulated mineral metabolism have been documented [4].

Importantly, experimental and clinical evidences indicate that vascular calcification (VC) plays an essential role in the development of cardiovascular diseases in CRF patients [5]. Calcification of the vascular smooth muscle cells (VSMC) results in increased vascular stiffness (VS) that causes systolic hypertension, left ventricular hypertrophy and reduced coronary perfusion. VC is an active, cell-mediated process that includes a.) increased uptake of calcium and phosphate by the VSMC, b.) transformation of VSMC to osteoblast/chondrocyte-like cells, c.) apoptotic cell death d.) release of VSMC-derived vesicles and e.) deficiency of circulating or local calcification inhibitors.

Recent studies suggest that the interaction between endothelium and VSMCs may also regulate the progression of VC. Activated and/or damaged endothelial cells in uremic patients with or without VC release great number of microparticles that promote the expression of osteogenic phenotype in endothelial progenitor cells, VSMCs and fibroblasts. The rise in endothelial microparticles is paralleled with a marked reduction in the endothelial progenitor cells, that are responsible for the repair of endothelium. Impaired endothelial progenitor cells may contribute to vascular dysfunction in patients with chronic kidney disease [6]. The apparent imbalance between endothelial damage and repair is claimed to contribute to the generation of VC [7].

Recent data suggest that impaired bone metabolism may have an important impact on the development of VC. This notion is supported by the observations that a/ bone loss and low bone mineral density is associated with increased VC and with increased cardiovascular events [8-10], b/ molecular mechanisms characteristic for bone formation have been observed at VC lesion levels [11, 12] and c/ the two conditions share several common risk factors including inflammation, oxidative stress and endothelial dysfunction that are known to promote VC and bone loss simultaneously [1].

The deleterious interaction between bone loss and VC results from the dysfunction of bone-vascular axis that is amplified in CRF. Numerous inhibitors and promoters have been identified that may play a role in mediating the cross talk between the skeletal and vascular systems [13]. Particular attention was paid to bone-related proteins including osteoprotegerin (OPG), osteocalcin (OC) and osteopontin (OP) [14-17].

VS is an increasingly recognized clinical marker of arteriosclerotic damage and it has been proved to be an independent predictor of fatal and non-fatal cardiovascular events in the general population, in elderly subjects and in patients with hypertension, diabetes mellitus and end-stage renal disease (ESRD) [18].

Furthermore, AS has been found to be associated with urinary creatinine excretion in chronic kidney disease [19] and renal damage has been shown to be related to AS and inflammation in patients with coronary artery disease [20].

The present study was undertaken in CRF patients receiving regular hemodialysis (HD) a/ to assess the extent and severity of arteriosclerotic lesion by measuring VS, b/ to reveal the possible association between AS parameters and OPG, OC and OP and c/ to determine the relationship of these selected bone-related proteins to some well-established clinical and biochemical risk factors of cardiovascular diseases.

Materials and Methods

Patients

68 (33 male, 35 female) consecutive chronic hemodialysis (HD) patients in stable clinical condition treated in the FMC Dialysis Center Pécs, Hungary were enrolled in this cross-sectional study. Patients having lower extremity amputation, any acute infection, malignancy, acute myocardial infarction, pulmonary edema or hemodynamic instability were excluded from the study.

The underlying renal pathologies that progressed to ESRD were the following: diabetic nephropathy (26%), benign nephrosclerosis (23%), chronic glomerulonephritis (15%), polycystic kidney disease (13%), chronic interstitial nephritis (10%), renovascular disease (1%) and other/unknown causes (12%). Most of the patients (66) received antihypertensive therapy.

The patients underwent 3 hemodialysis sessions per week, each having a duration of 4 hours. On-line hemodiafiltration was carried out by using Fresenius 5008 B equipment with Helixone/Fresenius polysulfone high-flux dialyzer membranes. Results presented are predialysis values. Body mass index (BMI) was calculated.

35 healthcare workers having no cardiovascular, metabolic and kidney disease served as controls for the analysis of cardiovascular and biochemical parameters.

Blood pressure and pulse wave velocity (PWV) measurements

Blood pressure was measured using calibrated automated devices with appropriate cuff sizes. The results presented here are predialysis values. Pulse pressure and mean arterial pressure was calculated.

Carotid-femoral PWV and augmentation index (AIx) was measured using applanation tonometry (SphygmoCor system, AtCor Medical Australia). Measurements were performed before hemodialysis sessions in supine position after at least 10-min rest in a quiet, temperature-controlled room. Measurements of the controls were performed in the morning in similar way. Pulse wave recordings were performed consecutively at two superficial artery sites (carotid-femoral segment). All readings recorded met the manufacturer's quality control standards integrated into the software package. The carotid-femoral PWV was calculated.

Laboratory measurements

Routine biochemical parameters were measured by standard methods. The serum concentrations of OC, OP and OPG were measured by Sandwich Enzyme-Linked Immunosorbent Assay (ELISA) (IBL International GmbH, Hamburg, Germany and BioVendor Laboratory Medicine Inc., Brno, Czech Republic). The respective intra-assay CVs were ranging from 4.7 to 5.5%; 4.4 to 10.1% and 2.5 to 4.9%, while the inter-assay CVs from 5.7 to 8.3%; 8.6 to 9.3% and 1.7 to 9.0%. (Table 1).

Statistical analysis

Statistical analyses were performed using the 21.0 software of the SPSS (SPSS, Inc., Chicago, IL, USA). Normality of distribution of data was tested by Kolmogorov-Smirnov test. Non-normally distributed parameters were transformed logarithmically to correct their skewed distributions. Correlations between continuous variables were assessed by calculation of linear regression using Pearson's test. Data were expressed as means $\pm$ S.D. in case of normal distribution, and median (lower/upper quartile) in case of non-normal distribution. Backward multiple regression analyses were performed to determine the relative contribution of selected independent variables in the constructed model to the variance of the dependent variable. Values of $P < 0.05$ were considered statistically significant.

Ethical considerations

The research was approved by the Regional Ethical Committee. The investigation conforms to the principles outlined in the World Medical Association Declaration of Helsinki. Informed written consent was obtained from all participants.

Table 1. Clinical and laboratory characteristics of 68 chronic hemodialysis patients and 35 controls. SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; AIx, augmentation index; PWV, carotid-femoral pulse wave velocity; BUN, blood urea nitrogen; spKt/V, single pool Kt/V; LDL cholesterol, low-density lipoprotein cholesterol; HDL cholesterol, high-density lipoprotein cholesterol; iPTH, intact parathyroid hormone, NA: not applicable, * $p < 0.05$

Clinical data	Hemodialysis patients	Controls	Biochemical data	Hemodialysis patients	Controls
Age (years)	59.7±13.3	60.3±16.2	Creatinine (μmol/l)	658.7±185.2	101.8±9.2*
Height (cm)	166.6±8.6	171.7±9.1	BUN (mmol/l)	17.5±3.8	5.8±1.8*
Body weight (kg)	73.6±17.7	75.2±19.1	spKt/V	1.7±0.32	NA
Body mass index (kg/m ²)	26.3±5.1	27.6±7.2	Hemoglobin (g/l)	113±11.1	140.8±6.2*
Dialysis vintage (months)	50.9±40.5	NA	Albumin (g/l)	42.1±4.6	41.8±5.2
SBP (mmHg)	121.2±21.7	124.1±24.2	Total cholesterol (mmol/l)	4.64±1.1	4.89±1.6
DBP (mmHg)	79.4±8.8	81.8±9.4	LDL cholesterol (mmol/l)	3.0±0.9	2.68±1.1
Pulse pressure (mmHg)	50.2±15.9	49.6±17.2	HDL cholesterol (mmol/l)	1.22±0.46	1.28±0.38
MAP (mmHg)	91.8±12.4	90.1±14.6	Triglyceride (mmol/l)	1.51±1.24	1.42±1.28
AIx	31.6±10.4	28.8±8.1	Sodium (mmol/l)	138.4±2.1	137.6±3.2
			Potassium (mmol/l)	4.7±0.6	4.1±0.4*
PWV (m/s)	11.5±3.4	8.6±2.8*	Calcium (mmol/l)	2.2±0.2	2.3±0.3
Central augmentation pressure (mmHg)	13.8±7.3	12.7±8.4	Phosphate (mmol/l)	1.6±0.4	1.1±0.2*
Central SBP (mmHg)	117.3±20.2	121.1±21.4	Alkaline phosphatase (IU/l)	114.0±43.1	96.6±18.9
Central DBP (mmHg)	79.2±9.3	82.1±10.2	iPTH (pmol/l)	58.6±23.9	2.2±1.8*
Central pulse pressure (mmHg)	41.5±13.9	40.1±14.8	Vitamin D ₃ (nmol/l)	7.7 (5.3; 12.79)	34.2 (7.8; 24.81)*
			Osteocalcin (ng/ml)	370.5±246.7	19.8±5.4*
			Osteopontin (ng/ml)	848.9 (697.5; 979.5)	216.7 (27.7-468.2)*
			Osteoprotegerin (pmol/l)	26.2±9.9	5.2±1.3*

Results

As shown in Table 1 PWV and AIx increased significantly in our CRF patients on HD compared to adults without major cardiovascular, renal and metabolic morbidities. Furthermore, serum levels of the bone-related proteins OC, OPG and OP were several times higher in our uremic patients than in our controls.

Univariate linear regression analyses to reveal the association of VS markers with the clinical and laboratory parameters have demonstrated that PWV correlated positively with age ($r = 0.411$, $p < 0.000$), whereas negatively with serum creatinine ($r = -0.412$, $p < 0.000$), urea nitrogen ($r = -0.427$, $p < 0.000$), phosphate ($r = -0.325$, $p < 0.007$), potassium ($r = -0.307$, $p < 0.011$) and OC ($r = -0.247$, $p < 0.049$). AIx was found to relate directly to central pulse pressure ($r = 0.405$, $p < 0.001$), augmentation pressure ($r = 0.800$, $p < 0.000$), systolic blood pressure ($r = 0.316$, $p < 0.000$) and inversely to body height ($r = -0.254$, $p < 0.036$), weight ($r = -0.277$, $p < 0.022$), heart rate ($r = -0.436$, $p < 0.000$) and urea nitrogen ($r = -0.321$, $p < 0.008$) (Table 2). PWV and AIx appeared to be independent of all other variables studied.

Out of bone-associated proteins, OC was positively correlated with serum creatinine ($r = 0.543$, $p < 0.000$), urea nitrogen ($r = 0.358$, $p < 0.004$), phosphate ($r = 0.471$, $p < 0.000$) alkaline phosphatase ($r = 0.375$, $p < 0.002$), iPTH ($r = 0.512$, $p < 0.000$), central systolic blood pressure ($r = 0.348$, $p < 0.005$) and the time spent on HD ($r = 0.255$, $p < 0.042$). OC, however, negatively correlated with PWV ($r = -0.247$, $p < 0.049$) (Fig. 1). OPG was positively associated with age ($r = 0.652$, $p < 0.000$), and negatively with BMI ($r = -0.313$, $p < 0.011$), body weight ($r = -0.371$, $p < 0.002$) and height ($r = -0.261$, $p < 0.03$) (Table 2). Importantly, there were no significant correlations between OP and any clinical/laboratory parameters routinely measured in uremia. One can assume that OC ($r = 0.282$, $p < 0.024$) and OPG ($r = 0.256$, $p < 0.040$) are related (Table 2) suggesting that these distinct bone-related proteins may interact in uremic patients receiving regular HD.

Table 2. Statistically significant correlations in univariate linear regression analysis. PWV, carotid-femoral pulse wave velocity; BUN, blood urea nitrogen; OC, osteocalcin; AIX, augmentation index; PP, pulse pressure; SBP, systolic blood pressure; HD, hemodialysis; OPG, osteoprotegerin; BMI, body mass index

Stiffness parameters			Bone-associated proteins		
	r	P		r	P
PWV-age	0.41	<0.000	OC-serum creatinine	0.543	<0.000
PWV-serum creatinine			OC-BUN	0.358	<0.004
PWV-BUN	-0.412	<0.000	OC-serum phosphate	0.471	<0.000
PWV-serum phosphate	-0.427	<0.000	OC-alkaline	0.375	<0.002
PWV-serum potassium	-0.325	<0.007	phosphatase		
PWV-OC	-0.307	<0.011	OC-iPTH	0.512	<0.000
AIX-central PP	-0.247	<0.049	OC-central SBP	0.348	<0.005
AIX-augmentation	0.405	<0.001	OC-time spent on HD	0.255	<0.042
pressure	0.800	<0.000	OC-PWV	-0.247	<0.049
AIX-SBP			OPG-age	0.652	<0.000
AIX-body weight	0.316	<0.000	OPG-BMI	-0.313	<0.011
AIX-body height	-0.277	<0.022	OPG-body weight	-0.371	<0.002
AIX-heart rate	-0.254	<0.036	OPG-body height	-0.261	<0.034
AIX-BUN	-0.436	<0.000	OP-OC	0.282	<0.024
	-0.321	<0.008	OP-OPG	0.256	<0.040

Fig. 1. Relationship of PWV to Osteocalcin (a), Osteopontin (b) and Osteoprotegerin (c).

Multiple linear regression models were applied to establish independent variables that have significant impact on bone-related proteins in our clinical setting. The variance of OC was negatively influenced by PWV ($\beta = -0.25$, $p < 0.029$) and BMI ($\beta = -0.26$, $p < 0.026$) and positively by systolic blood pressure ($\beta = 0.37$, $p < 0.001$) and hsCRP ($\beta = 0.23$, $p < 0.049$). OPG as dependent variable was found to correlate directly with age ($\beta = 0.69$, $p < 0.000$) and inversely with BMI ($\beta = -0.31$, $p < 0.001$). When the model was conducted with OP as dependent variable LDL-cholesterol proved to be the only significant factor associated with OP ($\beta = 0.25$, $p < 0.044$) (Table 3).

Further analysis by this multiple regression model disclosed that the variance of PWV as dependent variable was only affected by age ($\beta = 0.53$, $p < 0.001$), bone-related proteins had no significant contribution. AIX, the other measure of VS appeared to be independent of all clinical/biochemical parameters analyzed.

Discussion

The present study demonstrated that in CRF patients on regular HD, PWV as a marker of VS increased significantly and there was several-fold elevation in serum levels of bone-related proteins (OC, OPG, OP). As these proteins have been claimed to be implicated in the development of VC, their relationship to PWV was sought and only OC was found to correlate with PWV.

Unexpectedly, a paradoxical improvement was observed in this indirect measure of VC when PWV was analyzed as a function of retention of metabolic waste products (creatinine,

Table 3. Statistically significant correlations in multiple linear regression analyses. β : standardized regression coefficient; %: relative contribution to the variance, respectively; PWV, pulse wave velocity; SBP, systolic blood pressure; BMI, body mass index; CRP, C-reactive protein; LDL cholesterol, low-density lipoprotein cholesterol

		Variable			
		β	t	P	%
Osteocalcin as a dependent variable (Model 1 ($R^2=0.291$))	PWV	-0,25	-2,24	0,029	6,1
	SBP	0,37	3,25	0,001	11,5
	BMI	-0,26	-2,28	0,026	7,0
	CRP	0,23	2,00	0,049	5,8
Osteoprotegerin as a dependent variable (Model 1 ($R^2=0.557$))	Age	0,69	7,60	0,001	43,3
	BMI	-0,31	-3,54	0,001	9,6
Osteopontin as a dependent variable (Model 1 ($R^2=0.096$))	LDL cholesterol	0,25	2,05	0,044	6,0

urea nitrogen, phosphate, potassium and iPTH). We have no apparent explanation for this observation, however, it is unlikely that these substances provide vascular protection, but rather one can assume that low-grade inflammation and oxidative stress outperform other factors and/or are independently associated with vascular disease [1]. In this regard it is to be considered that both, inflammation and oxidative stress are highly prevalent in patients with CRF and have pivotal role in development of VC [2-5]. Moreover, they are further amplified by HD process and ageing [7, 18, 21, 22]. Our study confirmed the importance of age by demonstrating positive association between PWV and age that remained strongly significant after adjustment for relevant confounders.

Osteocalcin (OC)

OC, the product of the bone γ -carboxylglutamate protein (BGLAP) gene, is a non-collagenous matrix protein mostly expressed by bone-forming osteoblasts. It has 45-50 amino acid residues that undergo post-translational modifications by vitamin K-dependent γ -carboxylation. Depending on the extent of its carboxylation several isoforms are formed and released into the circulation that have different biological activities including the regulation of VC and atherosclerosis, as well as the modulation of insulin secretion and insulin sensitivity. Importantly, OC is also expressed in the calcified atherosclerotic lesion where it may modulate this process [16, 23-25].

In patients with impaired renal function plasma OC levels are markedly elevated due to increased bone turnover and decreased renal elimination [26, 27]. Conflicting results are available concerning plasma OC as a predictor of cardiovascular diseases and related mortality. In general, low circulating OC concentrations were found to be associated with an increased risk of cardiovascular diseases [16, 28, 29]. In agreement with our findings inverse relationship was also seen between the risk of cardiovascular events and plasma OC levels in patients on regular HD suggesting a vasculo-protective role for OC [26, 30, 31]. By contrast, large prospective and cross-sectional studies revealed U-shaped association of OC with fatal cardiovascular events [32, 33]. In a recent study convincing evidences have

been provided that OC may be implicated in the endothelial damage-related VC in patients with CRF. Activated endothelial cells in uremia are capable of producing microparticles that induce OC expression in endothelial progenitor cells, VSMCs and fibroblast. The imbalance in the process of endothelial damage and repair, therefore, may contribute to VC via increasing the number of OC-positive endothelial progenitor cells [34].

Osteoprotegerin (OPG)

OPG is a member of TNF receptor superfamily and binds to two ligands: RANKL (receptor activator of nuclear factor KB ligand), a critical cytokine for osteoclast differentiation, activation and survival, and TRAIL (TNF-related apoptosis-inducing ligand) involved in immune surveillance. It functions as a decoy receptor and blocks the effect of RANK/RANKL on bone resorption and prevents TRAIL-induced apoptosis [35-38]. OPG is expressed in a variety of tissues including vascular endothelial and VSMCs and its production by activated endothelial cells has been shown to be enhanced by pro-inflammatory cytokines (TNF- α , IL-1 β) [37]. Targeted deletion of OPG gene resulted not only in osteoporosis, but also in VC and the occurrence of multinuclear osteoclast-like cells within the vascular wall [38]. Accordingly, OPG has dual protective role; it promotes skeletal mineralization but prevents mineralization in vascular tissue [39].

Elevated circulating OPG levels have been reported in patients with cardiovascular and/or with chronic renal diseases and it was found to be independently associated with all-cause mortality and atherosclerosis [14, 15]. Similar findings were published in CRF patients on regular HD [40, 41] and in those receiving kidney transplantation [42]. However, there have been studies that failed to document such an association. Our own results are in line with these findings as PWV was unrelated to OPG but was positively associated with age and negatively with BMI. One can speculate that age and BMI overwhelmed the effects of OPG, or alternatively, OPG we measured was underphosphorylated but phosphorylated OPG is needed to achieve inhibition of VC [13]. It is still debated whether the expression of the elements of OPG/RANKL/RANK axis in the vascular lesion and the high levels of circulating OPG are an epiphenomenon or they have a counter-regulatory role to attenuate the calcification process [13].

Osteopontin (OP)

OP is a bone-specific sialoprotein that has three splice variants with several post-translational modifications. It is expressed in the mineralized bone matrix and various cells including macrophages, and vascular endothelial-, smooth muscle-, and epithelial cells. Due to the generation of a variety of OP forms with different bio-activity, OP is a multifunctional protein that regulates bone remodeling, inhibits ectopic calcification and is involved in inflammation, fibrosis and cell survival. It has been demonstrated to play a pivotal role in atherosclerotic vasculopathy by inducing inflammation and by inhibiting VC [17]. Clinical evidences have been provided that OP is associated with the extension and severity of coronary atherosclerosis and with adverse cardiac outcome in patients with ischemic heart disease. Furthermore, OP plasma levels were positively correlated with VS in patients with coronary disease but not in young subjects without clinical symptoms or risk factors of atherosclerosis [43, 44]. Interestingly, OP levels could be reduced by statins and angiotensin II blockade [45].

In patients with chronic kidney disease elevated circulating OP level has been reported but except for a small group of HD patients no independent association could be detected between OP level and VC [46, 47]. In experimental uremia plasma OP was increased and correlated positively with aortic atherosclerosis [48]. In our study we confirmed that plasma OP level is markedly increased in patients on regular HD but it does not relate to VS parameters. It is of note, however, that the risk factor LDL-cholesterol had independent influence on PWV.

Our study in patients receiving maintenance HD provided additional evidences that PWV as a measure of VS increased significantly and there was several-fold increase in serum levels of interrelated bone-specific proteins (OC, OPG, OP). PWV was found to be independently associated only with OC and age but risk factors for arterial calcification had significant impact on OC (systolic blood pressure, hsCRP, BMI), OPG (age, BMI) and OP (LDL-cholesterol).

The relatively small sample size in this study is a potential limitation. Furthermore, the cross-sectional study design could not confer the causal relationship between arterial stiffness, bone-related proteins and potential confounders. Longitudinal, prospective studies including detailed analysis of the impact of various etiology and medication on clinical and biochemical parameters are to be conducted in HD patients to draw definitive conclusion.

Conclusion

We could establish a role for OC in development of VC but our results failed to document direct association of vascular lesion with OP and OPG, therefore their high circulating levels may be an epiphenomenon or they may have counter-regulatory role to attenuate the uremic calcification process.

Disclosure Statement

The authors of this work declare that they do not have any Disclosure Statements.

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