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Short Communication

Excretion of anti-angiogenic proteins in patients with chronic allograft dysfunction

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Abstract

Background. We have recently documented the appearance of an anti-angiogenic peptide, endorepellin, in the urine of patients with chronic allograft dysfunction (CAD).
Methods. Here, we analyzed, using enzyme-linked immunosorbent assay, the excretion of anti-angiogenic peptides endostatin, pigment epithelium-derived factor (PEDF) and Kruppel-like factor-2 (KLF-2), in healthy individuals, patients with stable graft function and patients with various degrees of CAD.
Results. In healthy subjects and patients with CAD-0, endostatin, PEDF and KLF-2 excretions were at the level of detection. In contrast, there were significant differences between the patients with CAD-3 and CAD-0, CAD-1 and healthy controls for endostatin and CAD-0 versus CAD-3 for PEDF, but no differences in KLF-2 excretion. Receiver operating characteristic (ROC) curve analyses demonstrated a highly discriminative profile for all three biomarkers: the combination of these parameters offered 83% sensitivity and 90% specificity in distinguishing CAD-0 from CAD-1–3. The quality of these potential biomarkers of CAD was, however, highest in discriminating CAD status in biopsy-proven cases and dropped when CAD-0 was diagnosed based on clinical criteria.
Conclusions. In conclusion, these findings indicate the diagnostic potential of urinary detection of endostatin, PEDF and to lesser degree KLF-2 and suggest a mechanistic role played by anti-angiogenic substances in the developing vasculopathy and vascular rarefaction in patients with CAD.

Keywords: endostatin; pigment epithelium-derived factor; Kruppel-like factor 2

Introduction

Chronic allograft dysfunction (CAD) is the most prevalent cause of late renal graft loss. This heterogeneous disease cannot be reliably diagnosed using non-invasive techniques and the histological diagnosis as a variable combination of fibrosis, vascular and glomerular damage is considered a gold standard [1–5].

Our previous mass spectroscopy studies demonstrated the *de novo* appearance in the urine of CAD patients of anti-angiogenic peptides [6]. Since vasculopathy is considered one of the leading factors in the development of graft disease [7], finding of anti-angiogenic proteins spurred the interest in examining these proteins in greater detail in individual patients. We have recently documented the appearance of one anti-angiogenic peptide, endorepellin, in the urine of patients with CAD occurring at the expense of the parent molecule, perlecan [6]. Here, we analyzed the excretion of three additional anti-angiogenic peptides endostatin [8, 9], pigment epithelium-derived factor (PEDF) [10–18] and Kruppel-like factor-2 (KLF-2) [19–22], in healthy individuals, patients with stable graft function and patients with various degrees of CAD.

Materials and methods

Patient characterization

Patient populations participating in this study have been described in detail in our previous publication [18]. Recruitment was performed at Westchester Medical Center (WMC) and New York Presbyterian Hospital (NYPH). Patients in NYPH underwent protocol biopsies, in WMC, CAD-0 diagnosis was made clinically. All patients completed a written consent form which permitted both urine and data collection in compliance with the Health Insurance Portability and Accountability Act following protocol approval by the Institutional Review Boards for clinical trials involving human subjects.

Urine specimens were centrifuged at 2500 r.p.m (700 *g*) for 10 min, aliquoted and stored at −80°C without protease inhibitors, as detailed in [6].

Clinical data, including blood pressure and serum creatinine, were abstracted from the patient records and presented previously [6]. All cases of CAD-1–3 were biopsy confirmed. The type and severity of allograft pathologies were classified according to the Banff'-97 criteria [23] and 2007 classification.

Enzyme-linked immunosorbent assay of endostatin, PEDF and KLF-2

Endostatin and PEDF levels were quantified using the commercial Quantikine Human Endostatin ELISA kit (R&D Systems, Minneapolis, MN) and ChemiKine PEDF ELISA kit (Millipore, Temecula, CA), according to the manufacturers' instructions with minor modifications.

KLF-2 levels were measured using enzyme-linked immunosorbent assay (ELISA) developed in the laboratory, as detailed in Supplementary methods. Statistical analysis was performed as detailed in Supplementary methods.

Results

Results of ELISA detection of endostatin, PEDF and KLF-2 are summarized in Figures 1A–C and 2A–C. In healthy subjects and pooled patients with biopsy-confirmed IF/TA-0 combined with non-biopsied CAD-0, endostatin excretion was at the detection level. Kruskal–Wallis test (Table 1) showed that there were significant differences ($P < 0.05$)

among the groups (CAD-0 versus CAD-2 and CAD-3 for endostatin). PEDF excretion in healthy controls and combined IF/TA-0 and CAD-0 patients was at the detection level. Kruskal–Wallis test showed that there were significant differences ($P < 0.05$) only between the groups CAD-0

Table 1. Kruskal–Wallis comparison followed by Dunn's test for all the data obtained[a]

	Endostatin	PEDF	KLF-2
CAD-0 versus CAD-1	NS	NS	NS
CAD-0 versus CAD-2	*	NS	NS
CAD-0 versus CAD-3	*	*	NS
CAD-0 versus healthy	NS	NS	NS
CAD-1 versus CAD-2	NS	NS	NS
CAD-1 versus CAD-3	NS	NS	NS
CAD-1 versus healthy	NS	NS	NS
CAD-2 versus CAD-3	NS	NS	NS
CAD-2 versus healthy	NS	NS	NS
CAD-3 versus healthy	NS	*	NS

[a]The total Type I error alpha is 0.05 and each comparison is adjusted by Dunn's test rule. * means 'significant difference' and NS means 'not significant'.
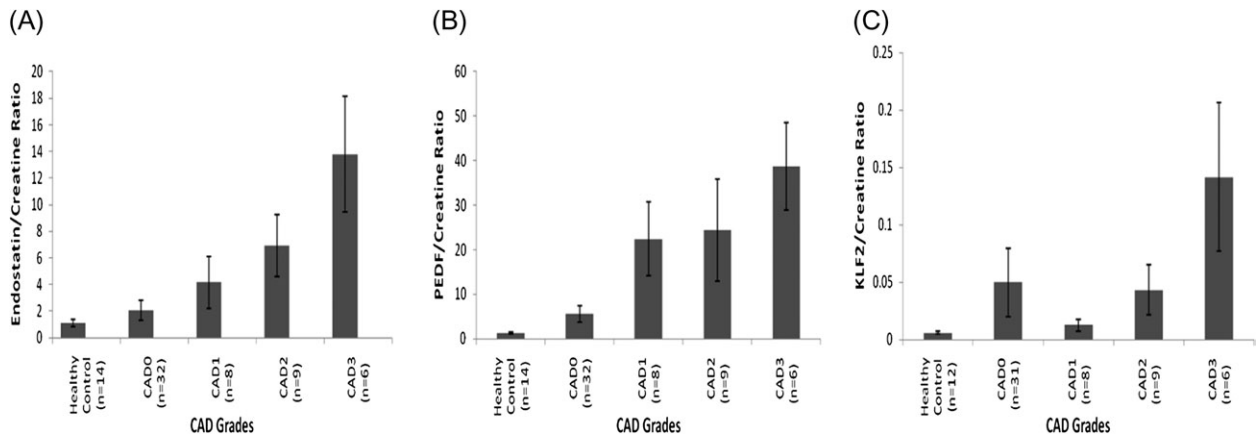

Fig. 1. (**A**) Endostatin, (**B**) PEDF and (**C**) KLF-2 urine concentrations, normalized using (urinary creatinine) in healthy non-transplant controls, in biopsy-confirmed and non-biopsy confirmed (CAD-0) and CAD-1, CAD-2 and CAD-3 patients. See statistical significance in Table 1.
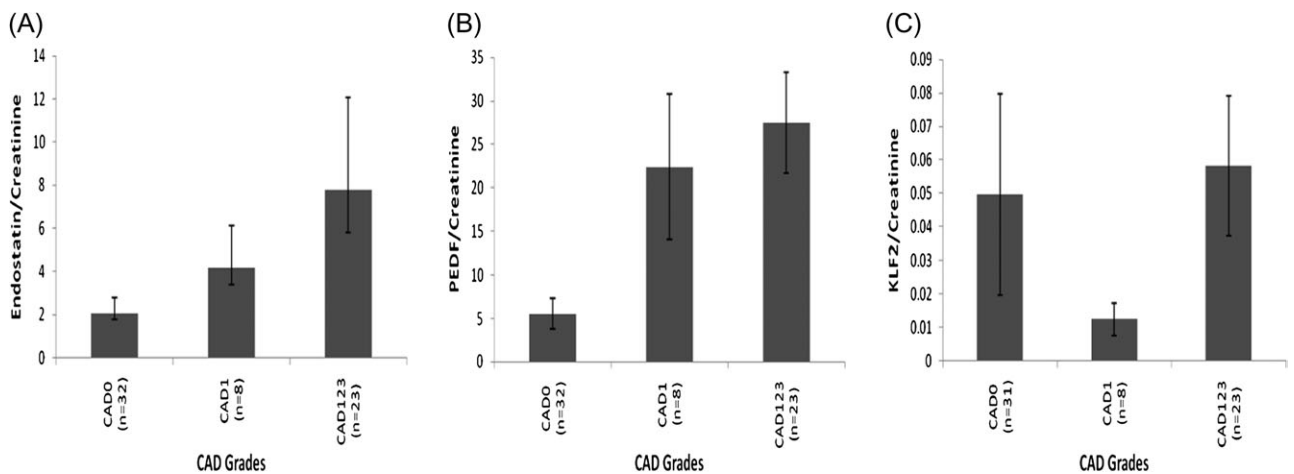

Fig. 2. (**A**) Endostatin, (**B**) PEDF and (**C**) KLF-2 urine concentrations, normalized using (urinary creatinine), in CAD patients (1–3), in biopsy-confirmed and non-biopsy confirmed CAD-0 and in healthy non-transplant controls.

versus CAD-3 (Table 1) KLF-2 excretion in healthy controls and combined IF/TA-0 and CAD-0 patients was also at the lower level of detection and Kruskal–Wallis test did not show difference among the groups. Regression analysis between endostatin, PEDF, KLF-2 and morphologic parameters is summarized in Table 2. Quartile analysis of data is presented in Supplementary figure 1.

Receiver operating characteristic curve analyses demonstrated that PEDF has the highest potential as a biomarker, with the area under the curve (AUC) = 0.828, in distinguishing patients with IF/TA-0 + CAD-0 from groups CAD-1–3 (Figure 3). Moreover, endostatin, PEDF and KLF-2 allowed the detection of conversion from the biopsy-proven IF/TA-0 to CAD-1 with

AUC of 0.861 (Figure 4). While these proteins poorly characterized clinically diagnosed CAD-0 (Supplementary figure 2 and Supplementary table 1), biopsy-confirmed IF/TA-0 showed a near perfect discrimination from CAD (Supplementary figure 3 and Supplementary table 2).

Discussion

Data presented herein demonstrated the validity of endostatin, PEDF and KLF-2 as potentially valuable candidate biomarkers of chronic allograft disease. The very fact that two of them are powerful anti-angiogenic substances (and the depletion of KLF-2 is also anti-angiogenic) nearly

Table 2. By two-group Wilcoxon test between endostatin, PEDF and KLF-2 levels and histological parameters of glomerular, tubulointerstitial and vascular disease (CG, CT, CV and CI) in CAD-1–3[a]

Parameter	Endostatin	PEDF	KLF-2
CG0:CG1	No patients	No patients	No patients
CG0:CG2	0.4724	0.9253	0.8644
CG0:CG3	0.2708	0.2405	0.5530
CT0:CT1	0.5116	0.0099	0.8414
CT0:CT2	0.0154	0.7330	0.8486
CT0:CT3	0.5214	0.1317	0.4685
CV0:CV1	0.8230	0.3523	0.7985
CV0:CV2	0.4617	0.0556	0.2313
CV0:CV3	0.3913	0.9644	0.8748
CI0:CI1	0.7157	0.0409	0.7638
CI0:CI2	0.0439	0.9279	0.8167
CI0:CI3	0.6165	0.1559	0.4942

[a]Regression analyses did not reveal any correlation between endostatin, PEDF or KLF-2 with the degree of renal dysfunction (as judged by serum creatinine concentration) or clinical parameters (data not shown). There was, however, significant correlation between endostatin, PEDF and KLF-2 levels in the urine. Analysis of correlation between endostatin, PEDF and KLF-2 levels and histological parameters of glomerular, tubulointerstitial and vascular disease in CAD-1–3 revealed a tight correlation between endostatin or PEDF with the severity of tubular and interstitial injury, but none showed correlation with the degree of glomerular involvement.
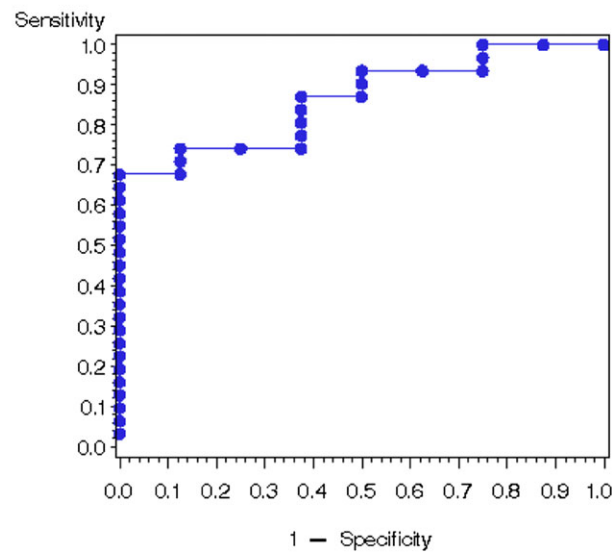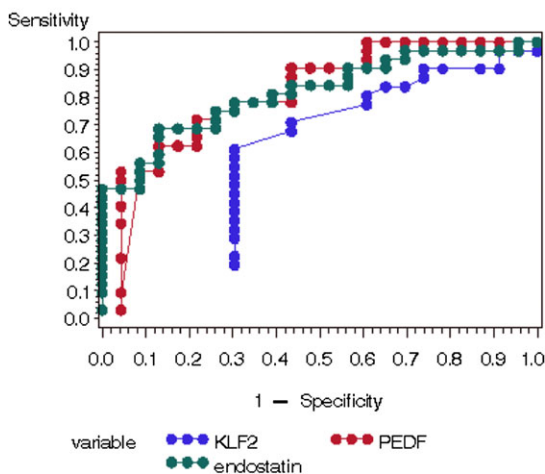

Fig. 4. Receiver operating characteristic curve analysis for CAD as its discrimination threshold is varied, in CAD-0 (biopsy-confirmed and non-biopsy-confirmed) versus CAD-1 patients.
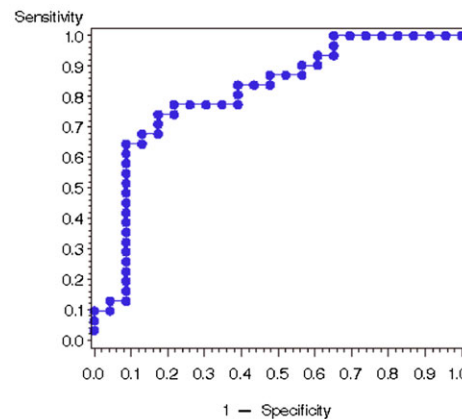

Fig. 3. Receiver operating characteristic curve analysis for CAD as its discrimination threshold is varied, in CAD-0 (biopsy-confirmed and non-biopsy confirmed) versus CAD-1, -2 and -3 patients.

absent in transplant recipients with normal graft function but appearing in the urine in association with developing CAD may have diagnostic and pathogenetic significance.

It is of significant interest to compare the findings obtained in biopsy-proven IF/TA-0 patients and those which were categorized similarly, but based only on clinical criteria. The diagnostic power of tests was much reduced when similar analyses were performed using CAD-0 diagnosis made exclusively based on clinical presentations. One of the future diagnostic strategies may consist in a dynamic monitoring of urinary levels of PEDF and endostatin (KLF-2 appears to be of a lesser importance) in the post-transplant monitoring and consider a biopsy, if levels surge.

Heart and kidney transplants are the most vulnerable organs for development of graft vascular disease [7]. Identification of the surge in anti-angiogenic and anti-endothelial PEDF and endostatin in the urine of kidney allograft recipients may have pathogenetic significance.

Future studies should focus on the prospective consecutive screening of individual anti-angiogenic substances in the samples of urine obtained from transplant patients and validating their diagnostic and prognostic value in patients with chronic allograft disease as diagnosed using protocol biopsy.

Supplementary data

Supplementary data are available online at http://ndt.oxfordjournals.org.

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