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ORIGINAL ARTICLE

Uremic Serum Induces Inflammation in Cultured Human Endothelial Cells and Triggers Vascular Repair Mechanisms

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Abstract— Inflammation and cardiovascular disease (CVD) are common in end-stage renal disease (ESRD) patients whose vascular endothelium is in direct contact with the uremic toxins found in the blood. These toxins are believed to affect vascular injury and repair process, which is impaired in ESRD patients. The exact mechanisms behind these interactions are not clear. So, we wanted to investigate what happens at the molecular level of endothelial cells when exposed to uremic serum from ESRD patients with diabetes and/or hypertension and its effect on the expression of molecules associated with vascular injury and repair. Cultured human endothelial cells (ECV304) were incubated in the presence of normal or uremic sera from ESRD patients with diabetes and/or hypertension. The expressions of monocyte chemoattractant protein 1 (MCP-1), vascular endothelial growth factor (VEGF), and stromal cell–derived factor 1 (SDF-1) were investigated in endothelial cells (ECV304) by real-time PCR and ELISA. The expression of MCP-1, VEGF, and SDF-1 was elevated in endothelial cells upon exposure to uremic sera from ESRD patients with diabetes and/or hypertension when compared with cells treated with healthy serum. MCP-1 expression in endothelial cells treated with uremic serum from ESRD patients with hypertension only was significantly increased compared with its expression in other cohorts. Exposure of endothelial cells to uremic serum causes endothelial injury and inflammation characterized by an increase in MCP-1 expression. This injury activates the initiation of vascular repair process in these cells by increasing the expression of VEGF and SDF-1. These molecules can be important biomarkers of chronic kidney disease–associated CVD.

KEY WORDS: uremia; endothelial cells; vascular injury; vascular repair; inflammation.

INTRODUCTION

End-stage renal disease (ESRD) is classified under stage 5 of chronic kidney disease (CKD) where glomerular filtration rate (GFR), an estimate of kidney function calculated as follows: $(186 \times (\text{creatinine}/88.4) - 1.154 \times (\text{age}) - 0.203 \times (0.742 \text{ if female}) \times (1.210 \text{ if black})$, is lower than 15 mL/min/m^2 of body area, or when patients are on

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dialysis regardless of their GFR [1]. Risk factors include older age (> 60 years), kidney disease history, obesity, diabetes, and hypertension. The last two are the leading causes of ESRD worldwide, are often associated with kidney disease as comorbidities, and contribute to its complications [2].

Inflammation and cardiovascular disease (CVD) are common features among chronic kidney disease (CKD) patients, especially at the advanced stages of the disease where the regulatory properties of vascular endothelium are altered [3], affecting its glycocalyx layer [4] and consequently interfering in vascular repair [5]. Endothelial dysfunction (ED) is characterized by the switch of the resting quiescent state of the endothelium to a proinflammatory state with prothrombotic properties and reduced vasodilation in response to stress and physical or chemical stimuli [6]. Upon injury and activation, endothelial cells release cytokines and chemokines into the blood stream such as monocytes chemoattractant protein 1 (MCP-1) [7], vascular endothelial growth factor (VEGF) [8], and stromal cell–derived factor 1 (SDF-1) [9]. MCP-1 secreted by endothelial cells attracts monocytes and macrophages to injured endothelium, playing a major role in the start and development of the inflammation process [10]. VEGF and SDF-1 attract endothelial progenitor cells (EPCs) from the bone marrow into the site of injury, where they adhere to activated endothelial cells, start the repair process, and eventually differentiate into new endothelial cells [11].

Whereas inflammation is augmented in end-stage renal disease patients [4, 12], vascular repair is severely impaired in those patients whose circulating EPCs were found to be numerically and functionally reduced [13]. The exact causes of this are not fully understood, but studies have shown that the overall systemic inflammation process observed in ESRD patients might be modulated by the interaction of uremic toxins found in their blood and endothelial cells, where uremic toxins contribute to vascular injury and impairs its repair [14, 15]. From this overview, we wanted to further investigate what happens at the molecular levels in the initial steps of the interaction between uremic serum and endothelial cells. Thus, the aim of this study was to investigate in the effect or uremic serum from ESRD patients on the expression of MCP-1, VEGF, and SDF-1, molecules associated with vascular injury and repair in an *in vitro* model of cultured endothelial cells. To further investigate the effects of comorbidities found in ESRD patients on this interaction, we divided the patients in this study into different cohorts with or without diabetes and/or hypertension.

MATERIALS AND METHODS

Patient Selection and Ethical Approval

Patient and healthy individuals were recruited at Orange Nassau Governmental Hospital in Tripoli, North Lebanon. The protocol was approved by the Ethics Committee in Human Research at Beirut Arab University and Lebanese University. Informed consent was obtained from all individual participants included in the study.

Control serum samples were obtained from 25 healthy donors and uremic serum samples were obtained from 147 patients on chronic hemodialysis. All participants were older than 18 years old. Exclusion criteria included active inflammatory signs; infectious disease or malignancies; autoimmune disease; active liver disease; prior or actual renal replacement therapy; the use of immunosuppressive drugs; and recent cardiovascular events (up to 3 months before the study began). Blood samples were collected before the initiation of the first dialysis session in the week and serum was combined together to 4 pools as follows: (1) patients with diabetes only ($n = 36$), (2) patients with hypertension only ($n = 40$), (3) those with both diabetes and hypertension ($n = 34$), and (4) those with neither ($n = 37$).

All samples (control and uremic) were obtained at the same time from donors/patients and stored under the same conditions at – 80 °C for few days until further processing.

Human Endothelial Cells Culture and Treatment

Human endothelial cells (ECV304) were obtained from Dr. Marwan Sabban at the American University of Beirut. Briefly, ECV304 cells were cultured at 37 °C with 5% CO_2 in modified Roswell Park Memorial Institute (RPMI-1640) media (Sigma-Aldrich) containing 20 Mm HEPES, L-glutamine, without sodium bicarbonate and supplemented with 10% (*v*/*v*) fetal bovine serum (FBS) (sigma-Aldrich), and 1% penicillin-streptomycin (Sigma-Aldrich). The media was replaced every 48 h until cells reached ~ 50% confluence. Afterwards, it was replaced daily and cells were subcultured when they reached 80–90% confluence. Once cells were confluent, they were transferred to experimental media consisting of culture media supplemented with 10% of control or uremic human serum. All experiments were performed using cells between passages 3 and 5.

Viability Assay by the Exclusion Method with Trypan Blue

The number of ECV304 cells was determined numerically by direct counting on a hemocytometer by the exclusion method with trypan blue (Sigma-Aldrich, USA). Cells were cultured and treated with uremic and healthy media under the same conditions mentioned above, trypsinized, and resuspended with 1 mL RPMI-1640. Then, 10 μL of suspension was added to 10 μL of a 0.4% solution and counted on a hemocytometer with the aid of a light microscope (Nikon, Tokyo, Japan). The cells with dye uptake were considered non-viable, and the number of viable cells was calculated by subtracting the number of non-viable cells from the total cell count [16].

Quantification of Gene Expression by Real-Time PCR

After treating endothelial cells with uremic or control media for up 24 h, total RNA was purified at different time intervals using a commercially available kit (GenElute Mammalian Total RNA Miniprep Kit; Sigma-Aldrich, USA). Concentration and purity of RNA was estimated using absorption spectrophotometry in a Nanodrop 2000 apparatus (Thermo Scientific). cDNA was synthesized using a commercial kit (iScriptTM cDNA Synthesis Kit, Bio-Rad) following the manufacturer instructions. Quantification of gene expression was performed using commercial SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich, USA). Each primer was tested for linearity and efficiency of amplification, which was always between 90 and 110%. Normalization and relative quantification were performed with the ΔΔCt method, using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as housekeeping gene.

The following primers were used:

GAPDH-F: 5′CCACTTTGTCAAGCTCATTTCCT3′,
GAPDH-R: 5′TCTCTTCCTCTTGTGCTCTTGCT3′;
VEGF-F: 5′AGGGCAGAATCATCACGAAGT-3′;
VEGF-R: 5′GCTGCGCTGATAGACATCCA3′;
SDF-1F: 5′-TGCCAGAGCCAACGTCAAG′;
SDF-1R: 5′CAGCCGGGCTACAATCTGAA′;
MCP-1F: 5′GCTGTGATCTTCAAGACCATTGTG3′;
MCP-1R: 5′TGGAATCCTGAACCCACTTCTG3′

Supernatant Levels of VEGF, SDF-1, and MCP-1 Proteins

The supernatants of treated endothelial cells were collected at different times (up to 24 h) of treatment and stored at −80 °C until processing. MCP-1, VEGF, and SDF-1 protein levels were measured by enzyme-linked immunosorbent assay (ELISA) sandwich using commercially available kits (Sigma-Aldrich, USA) according to the manufacturer protocol. The absorbance values were determined using a microplate reader (BioTek ELx800 Absorbance Microplate Reader, UK) at 570 nm.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism Software V.6.04 (USA). Data are presented as mean ± mean standard error (MSE). Unless stated otherwise, each experiment was repeated at least three times and was performed in triplicate. Comparison between three or more groups was performed using one-way ANOVA for parametric data and the Mann-Whitney test for the non-parametric data. Comparison between two groups was performed using Student's t test. Statistical significance was set at $p < 0.05$.

RESULTS

The main clinical and medical parameters of the 147 ESRD patients enrolled in this study and divided into 4 cohorts are summarized in Tables 1 and 2, consecutively.

Molecular Expression of VEGF, SDF-1, and MCP-1 in Endothelial Cells Upon Exposure to Uremic or Healthy Media

mRNA Expression in Endothelial Cells Treated with Uremic/Healthy Serum

Figure 1 shows the effect of the uremic serum from different ESRD cohorts on the expression of the mRNA of MCP-1, VEGF, and SDF-1 in ECV304 cells at different time points. The expression of all three genes was significantly increased in all cohorts at different time points when compared with cells treated with healthy serum. Although MCP-1 mRNA expression was higher in cells treated with uremic serum at all points when compared with cells treated with healthy serum, those treated with uremic serum from hypertensive patients showed the highest levels of MCP-1 mRNA expression at all time points. The peak of VEGF mRNA expression was at 6 h after treatment with uremic serum for all cohorts, then decreased after that. There was no significant difference in VEGF mRNA expression levels between cells treated with uremic serum from different cohorts at the same time point. SDF-1 mRNA expression showed a time-dependent

Table 1. Demographical Characteristics of ESRD Patients

Parameter	None ($n = 37$)	Diabetes ($n = 36$)	Hypertension ($n = 40$)	Both ($n = 34$)
Age (years)	47 ± 12.9	59 ± 13.5	50 ± 9.6	52 ± 16.2
Gender (% male)	45	59	50	56
Presence of family history (%)	9	0	20	22
Smokers (%)	36	44	20	22
Time in dialysis (years)	4.2 ± 1.9	3.1 ± 1.3	6.3 ± 2.4	5.2 ± 6.2
Duration of dialysis session (hours)	4 ± 0.4	4 ± 1.1	4 ± 0.3	4 ± 0.4
Primary cause of kidney disease (%)				
Diabetes (%)	0	100	0	100
Hypertension (%)	0	0	22	0
Chronic glomerulopathy (%)	67	0	78	0
Other (%)	33	0	0	0

Values expressed in mean ± SD

increase for all cohorts that peaked at 24 h after treatment with uremic serum when compared with cells treated with healthy serum.

Protein Expression in the Supernatant of Endothelial Cells Treated with Uremic/Healthy Serum

Figure 2 shows the protein expression of MCP-1, VEGF, and SDF-1 in the supernatant of ECV304 endothelial cells after treatment with uremic serum from diabetic, hypertensive, diabetic and hypertensive, and neither diabetic nor hypertensive ESRD patients compared with cells treated with healthy serum. The protein expression followed a similar pattern to their corresponding mRNA. MCP-1 protein expression was higher in endothelial cells treated with uremic serum from all cohorts when compared with cells treated with healthy serum. Cells treated with serum from hypertensive ESRD patients showed the highest MCP-1 protein expression levels at all time points. VEGF protein expression increased in all endothelial cells treated with uremic serum to reach its peak 6 h after treatment and then decreased after that when compared with cells treated with healthy serum. There was no significant change between VEGF protein expression between different cohorts. Similar to its mRNA expression, SDF-1 protein showed a time-dependent increase that peaked at 24 h after treatment for all cohorts when compared with cells treated with healthy serum.

DISCUSSION

Endothelial cells are the primary tissue-blood barrier and are in direct contact with blood/lymph and the circulating cells [17]. Therefore, the activation and dysfunction of this layer is the first critical step in the development of inflammation and cardiovascular diseases.

MCP-1 plays a major role in role in attracting monocytes to the site of injury and the onset of inflammation in ESRD patients [7, 18]. MCP-1 is also an important factor in the pathogenesis and progression of renal failure. Higher urinary MCP-1 concentrations were found in CKD patients and correlated with kidney damage [7]. Moreover,

Table 2. Medical Characteristics of ESRD Patients

Parameters	Normal ($n = 25$)	None ($n = 37$)	Diabetes ($n = 36$)	Hypertension ($n = 40$)	Both ($n = 34$)
Urea (before dialysis) (mg/dL)	7–20	149 ± 36.2	124.6 ± 37	134 ± 36.5	129.5 ± 33.8
Urea reduction rate (URR)	–	68 ± 7%	65 ± 6%	70 ± 9%	65 ± 10%
Creatinine (mmol/L)	60–110	109 ± 45	90 ± 28	110 ± 18	97 ± 27
Hemoglobin (g/L)	12–17	11.8 ± 1.6	10.4 ± 1.6	11.2 ± 1.5	10.6 ± 1.2
Leukocytes	4.5–11	7.2 ± 1.4	8.1 ± 1.4	7.2 ± 1.9	10.7 ± 5.9
Sodium (mM)	135–145	139 ± 1.7	139 ± 2.7	140 ± 1.9	137 ± 6.9
Potassium (mM)	3.5–5	5.6 ± 0.9	5 ± 0.9	5.5 ± 0.6	5.6 ± 0.8
Calcium (mg/dL)	85–102	89.2 ± 11.7	90 ± 8.7	94.5 ± 9.7	88.8 ± 7.4
Phosphate alkaline (IU/L)	44–147	95.4 ± 36.6	129 ± 57.5	155 ± 152.2	132.5 ± 59.7
SGPT (IU/L)	7–56	26.6 ± 4	23 ± 9	18 ± 5	20 ± 9

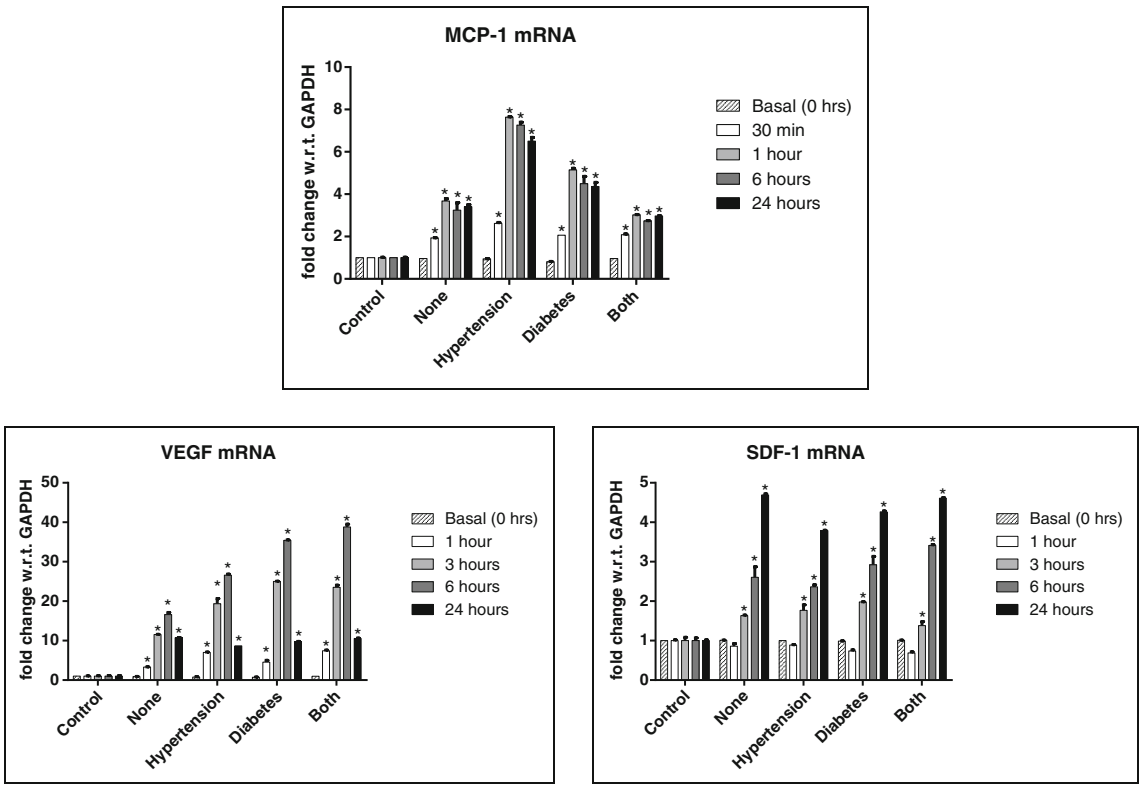

Fig. 1. mRNA expression levels in ECV304 cells after culture with uremic/healthy media from different cohorts at different time points. Time "0" represents the basal expression of mRNA levels **a** mRNA expression of MCP-1. **b** mRNA expression levels of VEGF. **c** mRNA expression of SDF-1. MCP-1 monocyte chemoattractant protein 1, VEGF vascular endothelial growth factor, SDF-1 stromal cell–derived factor 1.

mechanical injury of a confluent endothelial cells layer induced the secretion of MCP-1 by these cells *in vitro* [19]. We have found an increase in the expression of MCP-1 mRNA and protein in endothelial cells treated with uremic media when compared with control. This shows that the interaction of uremic serum with endothelial cells *in vitro* activated them and caused a response similar to that observed upon endothelial injury. The high levels of MCP-1 in ESRD patients are a reflection of inflammation observed in these patients. Interestingly, when the patients were divided according to their comorbidities, those with hypertension only had the highest levels of MCP-1. Hypertensive patients have an activated RAS and higher levels of angiotensin II in their blood. In fact, the inhibition of RAS reduced MCP1 expression in rats [20], and diabetes did not promote MCP-1 expression or augment it in hypertensive rats [21]. Thus, in addition to the initiation of endothelial inflammation caused by uremic serum and its toxins, the presence of comorbidities such as hypertension augmented this response.

VEGF and SDF-1 are released by endothelial cells upon activation, act as chemokines that recruit leukocytes to the site of endothelial injury, and take a role in the mobilization of EPCs from bone marrow [8, 19, 22]. Our results show that the exposure of endothelial cells to uremic serum *in vitro* caused an increase in the expression of these molecules. Although the high levels of these molecules could reflect a better endogenous repair system activity, the high incidence of cardiovascular disease in ESRD patients suggests that despite the production of VEGF and SDF-1 by the activated endothelium, vascular repair was severely impaired [23]. This is further supported by the low levels of circulating EPCs found in CKD patients [24] and the functional impairments of these cells that became more apparent as CKD progressed [25]. This implies that toxins found in uremic serum injured the cultured endothelial cells and provoked them to start the repair process by producing VEGF and SDF-1 to recruit EPCs from the bone marrow and that the impaired repair process is probably due to the functionally and numerically impaired EPCs rather than their chemoattracting

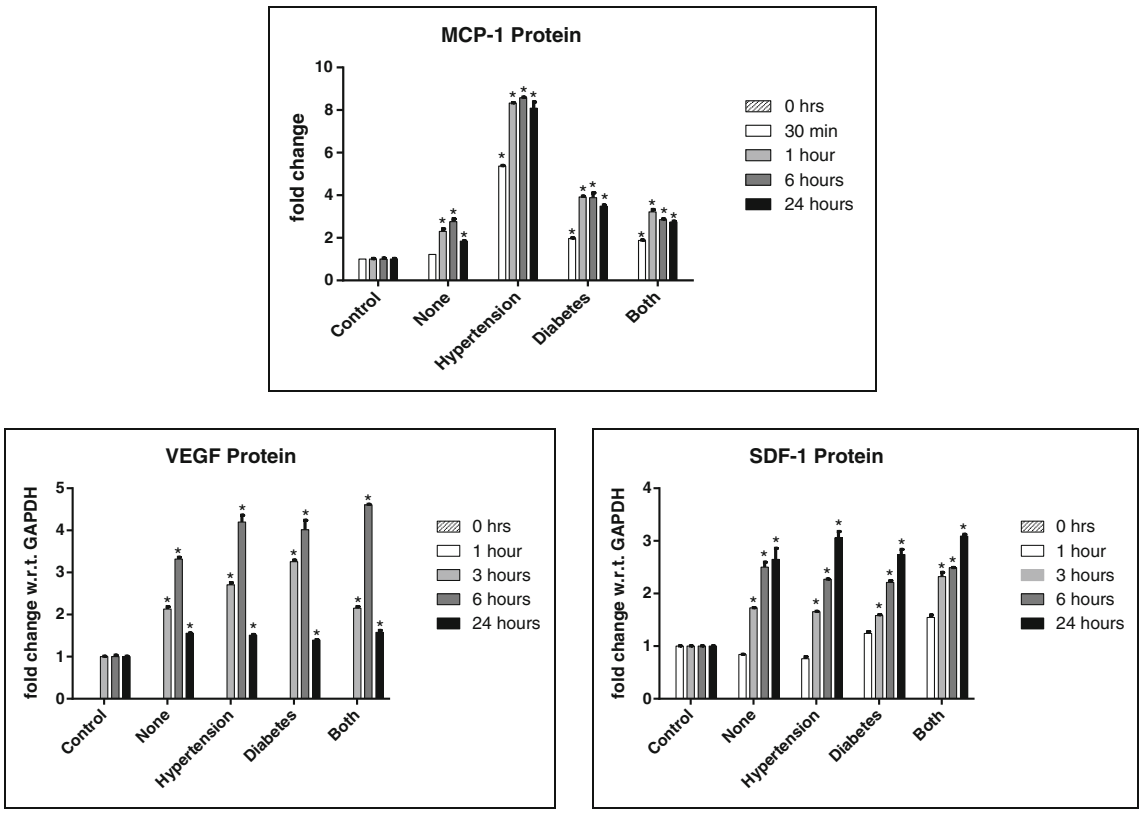

Fig. 2. Protein expression levels in ECV304 cells after culture with uremic/healthy media from different cohorts at different time points normalized to the expression of housekeeping gene GAPDH. **a** Protein expression levels of MCP-1. **b** Protein levels of VEGF. **c** Protein levels of SDF-1. MCP-1 monocyte chemoattractant protein 1, VEGF vascular endothelial growth factor, SDF-1 stromal cell–derived factor 1.

molecules. Our *in vitro* SDF-1 results contradict with another study that found a decrease in SDF-1 production by endothelial cells upon uremia exposure, although they found an increase in the *in vivo* SDF-1 levels in ESRD patients' serum [14]. We could not find the causes of this discrepancy in results, though our ELISA results are confirmed by the increase in mRNA production of SDF-1 upon uremic exposure, suggesting the effect of toxins in ESRD serum on injuring the endothelial layer and provoking its repair mechanisms.

In the last few years, several studies focused on the mechanisms of cardiovascular disease in ESRD patients [5, 12, 26], without looking into the association between these mechanisms and comorbidities found in ESRD patients such as diabetes and hypertension that are a major risk factor for CVD. We have found no difference in the expression of VEGF and SDF-1 in endothelial cells when treated with uremic sera from patients with or without diabetes and/or hypertension. The expression of these molecules by endothelial cells seems to be affected mainly by uremic toxins found in the blood of ESRD patients regardless of their comorbidities. Interestingly, in patients with diabetes or hypertension, high serum VEGF levels were only found in the presence of vascular diseases such as atherosclerosis and retinopathy [27, 28]. Moreover, in diabetic patients, the activity of soluble and cell surface dipeptidyl peptidase-4 (DPP-4), an enzyme that degrades SDF-1, was shown to be increased [22], affecting SDF-1 levels and subsequently vascular repair by EPCs in these patients. Our results are in agreement with other studies that found an increase in SDF-1 serum levels with deteriorating kidney function, even at the early stages of the disease [13] and that kidney transplantation restored EPCs levels and decreased serum SDF-1 [29]. It was also found that the renal clearance of SDF-1 is impaired in CKD patients [13]. We might say that high serum levels of SDF-1 observed in these patients are in a great part due to the increased expression of this molecule by activated endothelial cells, as shown by our results, and to the decrease of its removal by impaired kidneys in ESRD patients.

VEGF serum expression level in CKD patients is controversial in literature. Some have found an increase in VEGF serum levels in these patients [30], while others found a lower VEGF levels when compared with healthy controls [31, 32] and an increase in its excretion by defective kidneys [32]. Our results showed, for the first time, an increase in VEGF mRNA and protein expression by endothelial cells upon exposure to uremic serum that peaked at 6 h and decreased after that. All these findings combined suggest VEGF levels in ESRD patients are controlled by complicated mechanisms and need further investigation.

Our present results show that higher secretion of MCP-1, VEGF, and SDF-1 molecules correlates with their corresponding mRNA and that endothelial cells exhibit *de novo* expression of these molecules only after interacting with uremic sera. This was further supported by our findings of basal mRNA levels and nonexistent protein levels of these molecules before treatment with serum (at time 0). These experimental findings further implicate the effect of uremic toxins in the development and/or acceleration of endothelial inflammation and subsequently endothelial dysfunction and other vascular diseases observed in ESRD patients. This injury then provokes endothelial cells to start the repair process as a response by releasing VEGF and SDF-1. These findings stress the importance of chemokines production by endothelial cells as a major source of plasma levels and the role of these molecules as biomarkers in CKD-related CVD. Further studies should validate their role in predicting and monitoring cardiovascular outcome.

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COMPLIANCE WITH ETHICAL STANDARDS

All procedures performed were in accordance with the ethical standards of the institutional research committee at BAU and with the 1964 Helsinki declaration with its later amendments.

Conflict of Interest. The authors declare that they have no conflict of interest.

Informed Consent. Informed consent was obtained from all individual participants included in the study.

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