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Endothelial damage and vascular calcification in patients with chronic kidney disease

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Soriano S, Carmona A, Triviño F, Rodríguez M, Alvarez-Benito M, Martín-Malo A, Alvarez-Lara M-A, Ramírez R, Aljama P, Carracedo J. Endothelial damage and vascular calcification in patients with chronic kidney disease. *Am J Physiol Renal Physiol* 307: F1302–F1311, 2014. First published October 22, 2014; doi:10.1152/ajprenal.00114.2014.—Vascular calcification (VC) is a frequent complication of chronic kidney disease (CKD) and is a predictor of cardiovascular morbidity and mortality. In the present study, we investigated the potential involvement of endothelial microparticles (MPs) and endothelial progenitor cells (EPCs) in the generation of VC in CKD patients. The number of circulating EMPs is greater in patients with VC than without VC (307 ± 167 vs. 99 ± 75 EMPs/ μ l, $P < 0.001$). The percentage of EPCs is significantly lower in patient with VC than in patients without VC ($0.14 \pm 0.11\%$ vs. $0.25 \pm 0.18\%$, $P = 0.002$). The number of EPCs expressing osteocalcin (OCN) was higher in VC patients (349 ± 63 cells/100,000) than in non-VC patients (139 ± 75 cells/100,000, $P < 0.01$). In vitro, MPs obtained from CKD patients were able to induce OCN expression in EPCs from healthy donors; the increase in OCN expression was more accentuated if MPs were obtained from CKD patients with VC. MPs from CKD patients also induced OCN expression in vascular smooth muscle cells and fibroblasts. In CKD patients, the rise in endothelial MPs associated with a decrease in the number of EPCs, suggesting an imbalance in the processes of endothelial damage and repair in CKD patients, mainly those with VC. Our results suggest that EPCs, through OCN expression, may directly participate in the process of VC.

chronic kidney disease; endothelial damage; endothelial microparticles; endothelial progenitors cells; vascular calcification

VASCULAR CALCIFICATION (VC) is a frequent problem among patients with chronic kidney disease (CKD) (27). Its prevalence increases as renal function progressively deteriorates. VC is an independent and strong predictor of cardiovascular risk (15), and its clinical diagnosis is exclusively radiological since there no sufficiently sensitive and specific biomarker of VC (1, 6, 15, 27, 29, 32). Several radiological methods, such as

electron beam computed tomography, multislice spiral computed tomography, and plain radiographs, have been used to investigate aortic calcification. Such methods are expensive, and none of them have been accepted as the gold standard in cardiovascular risk assessment.

The integrity of the endothelium plays a key role in the pathogenic of vascular disease, including the development of VC (6, 29). Recent evidence has demonstrated that soluble factors secreted by endothelial cells regulate procalcific activity in vascular smooth muscle cells (VSMCs) (19, 35, 42). It has recently been shown that mature endothelial cells are capable of producing microparticles (MPs) in response to cellular activation or apoptosis (3, 4, 7, 25), a situation that has also been observed in uremia (12, 33).

To maintain vascular integrity, damaged endothelial cells are replaced by endothelial progenitor cells (EPCs). A function that has been ascribed to endothelial MPs (EMPs) is the ability to recruit and differentiate EPCs (5). In the clinical setting, changes in both the number and functions of EPCs have been positively related to an improved capacity for endothelial regeneration, which is inversely associated with cardiovascular risk (20, 34, 37, 38, 39, 43). EPCs can be identified not only through their angiogenic capacity but also by their expression of cell surface markers (14, 21, 30), and it has recently been reported that in patients with coronary atherosclerosis, a high proportion of EPCs may present an osteoblast-like phenotype (8, 9, 10, 28).

Our hypothesis was that in CKD patients, the cardiovascular disease includes damage of the endothelium, causing an increase in circulating EMPs and abnormalities of EPCs. Both processes may favor the development of VC in CKD. The aim of the present study was to 1) evaluate the potential association of VC in CKD with the number of MPs and with the number and function of EPCs and 2) investigate in vitro whether MPs and EPCs from CKD patients may be directly involved in the genesis of VC.

In the present study, EMPs and subpopulations of EPCs, including those with osteoblastic characteristics, were obtained from CKD patients with and without VC. Subsequently, the in vitro effect of MPs on the expression of osteogenic protein in endothelial cells, VSMCs, monocytes, and fibroblasts was investigated. EPC function was also evaluated.

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MATERIALS AND METHODS

Patients. Fifty-seven patients with CKD at stages 3–5 were selected from the Reina Sofia University Hospital (Cordoba, Spain). Patients with severe cardiovascular disease, active infections, or severe secondary hyperparathyroidism [parathyroid hormone (PTH) > 500 pg/ml] were excluded. The assessment of VC was performed by an original method similar to the Kauppila index (26, 41); in our patients, the presence of calcium deposits was assessed by abdominal computed tomographic angiography (CTA) of the aortal-abdominal region, and this technique is routinely performed as part of the evaluation for renal transplantation. We wanted to prove our own method and therefore measured abdominal aortic calcification by lateral lumbar X-ray (Kauppila index) in 26 of our patients that also had CTA. The Kauppila index was obtained by two highly experienced independent examiners. A radiologist with experience in the CTA technique developed a severity scoring system. Lateral lumbar spine radiographs were acquired, and a deposit index was developed to grade the severity of calcification in the aorta at the level of the first through fourth lumbar vertebrae. A separate score was determined for the anterior and posterior aorta, and the values were added across the four vertebrae, resulting in a calcification index that ranged from 0 to 24 points. We observed that the Kauppila index correlated highly with calcification scores from CTA ($r = 0.7$, $P < 0.001$). We consider that the method used in our study is as accurate as the Kauppila index.

Patients were divided into two groups on the basis of their median score from the first to the third lumbar vertebra. *Group 1* (without VC) included 29 patients with scores below 8.5, whereas 28 patients were assigned to *group 2* (score > 8.5, with VC). We recorded all clinical data could be related to VC: age, sex, body mass index, previous cardiovascular disease/events, arterial hypertension, diabetes mellitus, smoking habits, and treatment with statins, recombinant human erythropoietin, paricalcitol, and calcium- or noncalcium-containing phosphate binders. Arterial hypertension was defined as blood pressure above 140/90 mmHg or requiring antihypertensive treatment. All routine laboratory measurements, including high-sensitivity CRP, were made using certified assay methods in the clinical laboratory of Reina Sofia Hospital. Intact PTH levels were measured using an immunoradiometric assay (Quest Diagnostics Nichols Institute, San Juan Capistrano, CA). Triglycerides, total cholesterol in plasma, and lipoprotein fractions were assayed by enzymatic procedures. Smoking habits were assessed according to the current smoking status. Ten healthy subjects, matched for age and sex, served as controls. The Hospital Ethics Committee approved the study, and all subjects provided written informed consent.

Determination of CD31⁺/annexin V⁺ EMPs in plasma. Platelet-free plasma was obtained by centrifugation at 1,500 g for 10 min at room temperature followed by an additional centrifugation at 13,000 g for 20 min to separate EMPs, which were stored at -20°C until use. EMPs were then resuspended and incubated with monoclonal antibody against phycoerythrin (PE)-labeled anti-CD31 (Caltag Laboratories, Burlingame, CA) followed by an incubation with FITC-annexin V kits according to the manufacturer's instructions (Bender MedSystems). The negative control was obtained using anti-isotype antibodies. An equal volume of Flow Count Calibrator beads (Beckman Coulter, Marseilles, France) was added. Fluorescence-activated cell sorter analysis was performed in a Coulter Cytomic FC 500 flow cytometer (Beckman Coulter, Fullerton, CA) using CXP software (Beckman Coulter). Each result (single value) was the average of three independent determinations of the same sample.

Determination of EPCs in peripheral blood. A sample of 10 ml of peripheral venous blood was drawn from patients and healthy subjects into tubes containing lithium heparin. To identify EPCs, sample blood was incubated against PE-labeled anti-CD133 antibody (AC133 PE, MACS Miltenyc Biotec, Bergisch Gladbach, Germany), PE-cyanin 7-labeled anti-CD34 (no. 25-0349-42, eBioscience, San Diego, CA), and FITC-labeled anti-VEGF receptor 2 (VEGFR2; FAB357F, R&D

Systems Europe, Abingdon, UK). Immediately after blood extraction, whole blood was incubated for 30 min in the dark at 4°C with monoclonal antibodies or the corresponding isotype controls. Erythrocytes were subsequently lysed with 450 μl FACS Lysing solution (BD Biosciences) for 10 min at room temperature in darkness. Cells were then washed in PBS with 2% human serum albumin before flow cytometry analysis (Beckman Coulter). The number of EPCs was defined as events that were triple positive for CD133, CD34, and VEGFR2. Cells expressing these three molecules are the most commonly reported as "classical" EPCs (14, 43), but the differential expression of these molecules has been linked to different states of cellular differentiation as well as to the intensity of their repair capacity (14). We then evaluated three differentiation stages in EPCs in relation to the expression or absence of the molecules marked. The negative control (0 value) was the value obtained using isotype antibodies. Each result (one single value) was the average of three independent determinations of each sample.

Determination of osteogenic-related molecules in EPCs. At the same time as the triple labeling procedure described above, a primary antibody consisting of a mouse anti-human osteocalcin (OCN) monoclonal antibody or the corresponding control isotype was used at the manufacturer's recommended dosage. After a wash of the primary antibodies, cells were incubated with anti-isotype peridinin chlorophyll protein complex-conjugated secondary antibodies at 4°C for 60 min. All antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Purification and cultures of healthy EPC donors. Peripheral blood mononuclear cells of healthy donors were isolated by density gradient centrifugation (Lymphoprep, Axis-Shield PoC, Oslo, Norway), washed with PBS (GIBCO, Invitrogen, Carlsbad, CA) + 20% FBS, and resuspended in endothelial cell basal medium (EBM)-2 (Lonza, Allendale, NJ) supplemented with Single Quots endothelial cell growth medium (EGM)-2 (VEGF, human EGF-B, recombinant IGF-1, human EGF, heparin, ascorbic acid, and GA-100, Lonza) and 15% autologous plasma. Mononuclear cells were plated on fibronectin (Biocoat, BD Biosciences, Franklin Lakes, NJ) in coated six-well plates at a density of 5×10^6 cells/well. We then incubated the fibronectin plates at 37°C and 5% CO_2 ; 4 days later, we removed the cells in suspension, and the fraction of cells attached was cultivated with EBM-2 complemented by 20% PBS and 15% autologous plasma. This medium recharging process was repeated every 2 days for 3–4 wk. After about 3 wk of culture, EPCs could be visualized in an optical microscope (Motic AE30, Fujian, China) in the form of colonies (colony-forming units). The EPC phenotype (CD34⁺CD133⁺VEGFR2⁺) was verified, with a cellular purification of >90%.

Cultures of EPCs from healthy donors with EMPs. During the second week of EPC cultures, EMPs obtained from patients with or without VC were added. The concentration of EMPs had been

Table 1. Clinical data and pharmacological treatment

	VC Group	Non-VC Group	P Value
Number of patients/group	28	29	
Age, yr	67.4 \pm 5.3	62.3 \pm 8.7	0.06
Men/women, %	69.7/30.3	81/19	0.43
Body mass index	29.6 \pm 4.9	30.2 \pm 9.4	0.7
Cardiovascular history, %	42	21.6	0.04
Hypertension, %	84	75	0.07
Diabetes mellitus, %	30	21.6	0.08
Smokers, %	46.4	37.9	0.24
Statins, %	63	48	0.43
Paricalcitol, %	39.4	5.4	0.001
Phosphate binders, %			
Calcium carbonate	36.3	32.4	0.4
Lanthanum	21.2	46	0.02

VC, vascular calcification.

Table 2. Laboratory characteristics

	VC Group	Non-VC Group	P Value
Number of patients/group	28	29	
Creatinine clearance (MDRD-7)	15.6 ± 4.2	14.4 ± 3.5	0.20
Calcium, mg/dl*	9.3 ± 0.5	9.2 ± 0.7	0.41
Phosphate, mg/dl	4.5 ± 0.6	4.7 ± 0.8	0.16
25-Hydroxyvitamin D, ng/ml	23 ± 10	20 ± 8.7	0.25
Total cholesterol, mg/dl	190 ± 42	178 ± 34	0.62
High-density lipoprotein-cholesterol, mg/dl	34 ± 17	39 ± 20	0.8
Low-density lipoprotein-cholesterol, mg/dl	71 ± 39	84 ± 42	0.45
Triglycerides, mg/dl	160 ± 56	178 ± 43	0.08
Hemoglobin, g/dl	11.9 ± 0.4	12 ± 0.3	0.06
Albumin, g/dl	3.8 ± 0.4	4 ± 0.9	0.24
Intact parathyroid hormone, pg/ml	231 ± 120	156 ± 79	0.003
CRP, log(mg/l)	0.73 ± 0.4	0.45 ± 0.37	0.005

*Corrected serum calcium (in mg/dl): serum calcium (in mg/dl) + 0.8[4-serum albumin(in g/dl)].

previously calculated to be 5×10^5 EMPs· μl^{-1} ·well $^{-1}$. One well that contain EMPs from healthy subjects was used as a control. After 3 wk, colony-forming units were quantified, and cells were harvested from cultures as described above. The EPC phenotype was assessed by flow cytometry.

Capillary-like tube formation assay. Ninety-six-well plates (Corning Costar, Cambridge, MA) were coated with 50 μl Matrigel (BD Biosciences) and incubated at 37°C for 30 min. EPCs were trans-

planted to 96-well plates at a density of 1×10^5 cells/well. EMPs from 500 μl of plasma obtained from plasma patients with no VC and VC dissolved in low-serum medium (1% FBS) were added to each well. After 4 h of incubation, tube morphology was visualized in an optical microscope (Motic AE30). Each experiment was repeated in triplicate, and the tube length was assessed by ImageJ. The analysis consisted of a segmentation of the cell areas. The meshed area could be identified by looking for empty regions of the field delimited by tubules and cell clusters, and Nb segments are the elements delimited by two junctions.

Cell line cultures with MPs. Human umbilical vein endothelial cells were obtained from Cell Systems (Cell Systems/Clonetics, Solingen, Germany) and cultured with standard EBM (Cambrex Bio Science, Walkersville, MD), EGM supplements (Cambrex), and 10% FBS (GIBCO). Cells were detached using trypsin-EDTA (Lonza). Cells from a human monocytic leukemia cell line (THP-1 cells) were cultured in RPMI-1640 without L-glutamine (Lonza) supplemented with 0.1% nonessential amino acids, 100 U/ml penicillin, 100 mg/ml streptomycin, and 20% FBS. Human aortic smooth muscle cells (VSMCs) were obtained from Clonetics and were cultured in DMEM supplemented with FBS (20%), Na pyruvate (1 mM), glutamine (4.5 g/l), penicillin (100 U/ml), streptomycin (100 mg/ml), and HEPES (20 mM) at 37°C in a humidified atmosphere with 5% CO₂. Cells were used after the fifth passage. Normal human dermal fibroblasts, isolated from the dermis of juvenile foreskin or adult skin, were obtained commercially (PromoCell, Heidelberg, Germany) and cultured in a Fibroblast Growth Medium kit (PromoCell). Cells were incubated in DMEM (GIBCO) supplemented with 10% FBS at 37°C and 5% CO₂. Other reagents were purchased from Sigma-Aldrich.

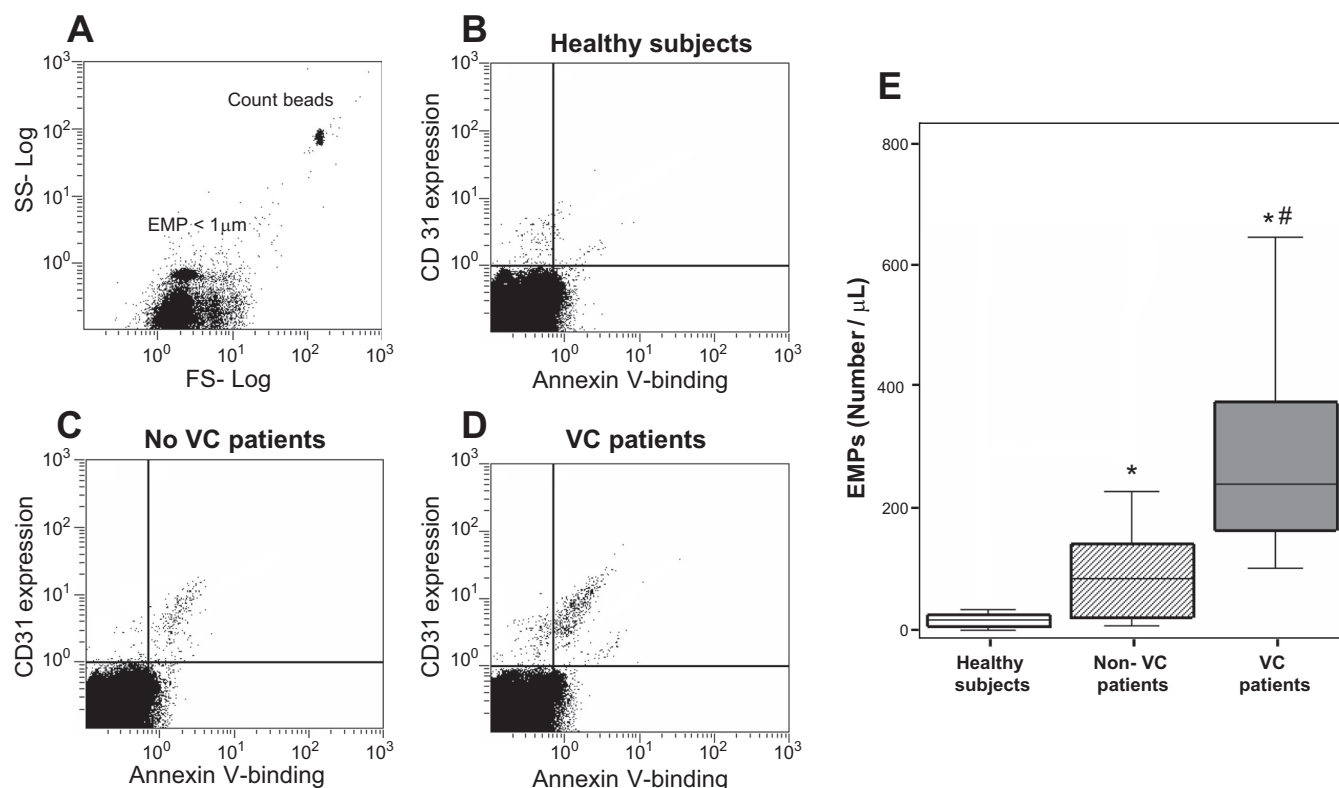


Fig. 1. Quantification of endothelial microparticles (EMPs). Representative graphs of flow cytometry analysis of EMPs in platelet-free plasma from healthy subjects, chronic kidney disease (CKD) patients without vascular calcification (non-VC), and CKD patients with vascular calcification (VC) are shown. A: EMPs were plotted using a forward scatter logarithmic (FS- log)/side scatter logarithmic (SS- log) dot-plot histogram. EMPs are defined as event numbers with a size of 0.1–1 μm and are gated in a window. It was necessary to use bead counts (circled area) in each experiment to calculate the concentration of EMPs per unit volume of sample. B–D: size-selected events plotted as a function of their double fluorescence for specific annexin-phycoerythrin (PE) binding and CD31-FITC. E: the number of EMPs was quantified in each of the three groups (healthy subjects, non-VC patients, and VC patients) as shown in the box diagram. * $P < 0.001$ vs. healthy subjects. # $P < 0.001$ vs. non-VC patients.

MPs obtained from patients or control subjects were added to the cell line cultures under the same conditions described above. The expression of OCN was quantified after 1 wk of culture.

Statistical analysis. Data normally distributed are presented as medians and interquartile ranges. Normally distributed data are shown as means \pm SD. The Wilcoxon rank-sum test was performed on continuous variables, and dichotomous variables were compared using Fisher's exact test. ANOVA was used to test differences of means among VC, non-VC, and healthy subjects. Skewed data were transformed using a log transformation. Spearman correlation coefficient was used to describe relationships between EMP and EPCs. *P* values of <0.05 were considered statistically significant. The statistical package used was SPSS (version 17).

RESULTS

Patient characteristics. The relevant clinical information and patient's treatment are shown in Table 1. Patients with VC were not significantly older than non-VC patients, and there were more men in both groups. We did not observe a prevalence of any particular pathology with regard to the origin of CKD in VC subjects compared with non-VC subjects. More cardiovascular events were noted in patients with VC (42%) than in non-VC patients (22%, $P = 0.04$). Treatment with paricalcitol predominated in patients diagnosed with VC (39.3% in the VC group vs. 5.4% in the non-VC group, $P = 0.001$), and treatment with lanthanum carbonate predominated in the non-VC group (46% vs. 21% in the VC group, $P = 0.02$).

Biochemical data are shown in Table 2. There were no significant differences in any of the parameters evaluated.

Renal function (MDRD-7) and the degree of anemia were similar in both groups. Lipids and parameters of mineral metabolism were also similar in both groups except for PTH, which was higher in VC than non-VC patients. Interestingly, high-sensitivity CRP levels were higher in VC than non-VC subjects (0.73 ± 0.4 vs. 0.45 ± 0.37 mg/l, $P = 0.005$).

Evaluation of CD31⁺/annexin V⁺ EMPs. Representative graphs of flow cytometry analysis of EMPs and the number of EMPs (CD31⁺/annexin V⁺) in CKD patients are shown in Fig. 1, A–E. Samples from VC patients showed an increased number of EMPs compared with non-VC patients (307 ± 167 vs. 99 ± 75 EMPs/ μ l, $P < 0.001$). In both groups of patients, the number of EMPs was greater than in healthy subjects (26.9 ± 12.8 EMPs/ μ l, $P < 0.001$).

Quantification of EPCs. The capacity to repair damaged endothelium is related with the amount of EPCs. The EPC phenotype was identified based on combined triple fluorescence for CD34, CD133, and VEGFR2 as analyzed by flow cytometry (Fig. 2). In VC patients, the percentage of EPCs was significantly lower than in non-VC patients ($0.14 \pm 0.11\%$ vs. $0.25 \pm 0.18\%$, $P = 0.002$; Fig. 2C). It should be emphasized that both groups of CKD patients, with and without VC, had a lower proportion of EPCs than the control group (1.85 ± 0.6 , $P < 0.001$). Interestingly, in CKD patients, EMPs correlated negatively with EPCs ($r = -0.37$, $P = 0.003$).

We identified EPCs that were CD34⁺ and CD133⁺VEGFR2⁺; these were defined as "early" and highly active EPCs. CD34⁺CD133⁺VEGFR2⁺ and CD34⁺CD133⁻VEGFR2⁺ EPCs are more highly differentiated in terms of CD133 expression.

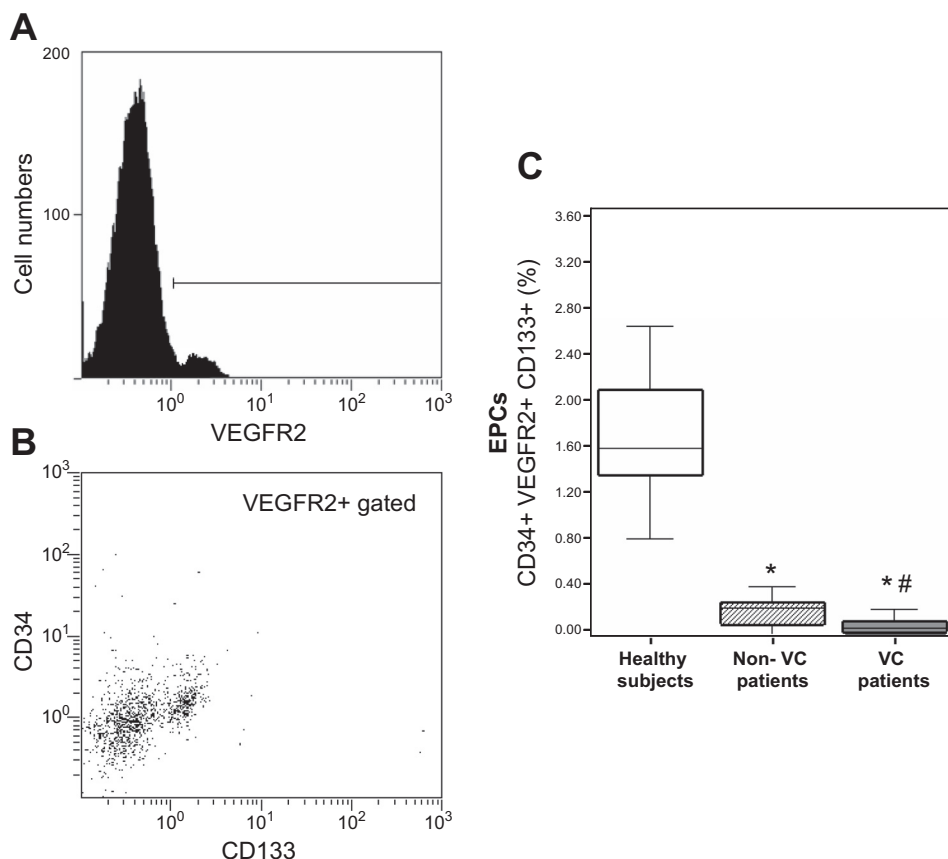


Fig. 2. Quantification of endothelial progenitor cells (EPCs). Representative graphs of cytofluorometric analyses of triple-positive EPCs [CD34⁺VEGF receptor 2 (VEGFR2)⁺CD133⁺] in the blood of healthy subjects are shown. A and B: VEGFR2⁺ cells were selected (A) and CD34⁺CD133⁺ double-positive cells were then quantified in VEGFR2⁺ cell gate (B). quantification of EPCs in healthy subjects, non-VC patients, and VC patients. * $P < 0.001$ vs. healthy subjects; # $P = 0.002$ vs. non-VC patients.

Table 3. EPC quantification in peripheral blood from chronic kidney disease patients and OCN expression in EPC subpopulations

	CD34 ⁻ CD133 ⁺ VEGFR2 ⁺		CD34 ⁺ CD133 ⁺ VEGFR2 ⁺		CD34 ⁺ CD133 ⁻ VEGFR2 ⁺	
	Total, %	OCN ⁺ cells/100,000	Total, %	OCN ⁺ cells/100,000	Total, %	OCN ⁺ cells/100,000
Healthy subjects	0.86 ± 0.2	7.9 ± 3.5	1.85 ± 0.6	15.1 ± 9.2	3.91 ± 1.7	34 ± 8.4
VC patients	0.05 ± 0.01*†	349.3 ± 63*†	0.14 ± 0.1*†	39 ± 9.3*	1.93 ± 1.2*	225.6 ± 87*
Non-VC patients	0.12 ± 0.09*	139.2 ± 75*	0.25 ± 0.18*	28 ± 12*	2.45 ± 1.1*	117 ± 44*

EPCs, endothelial progenitor cells; VEGFR2, VEGF receptor 2; OCN, osteocalcin. **P* < 0.01 vs. controls; †*P* < 0.01 vs. non-VC patients.

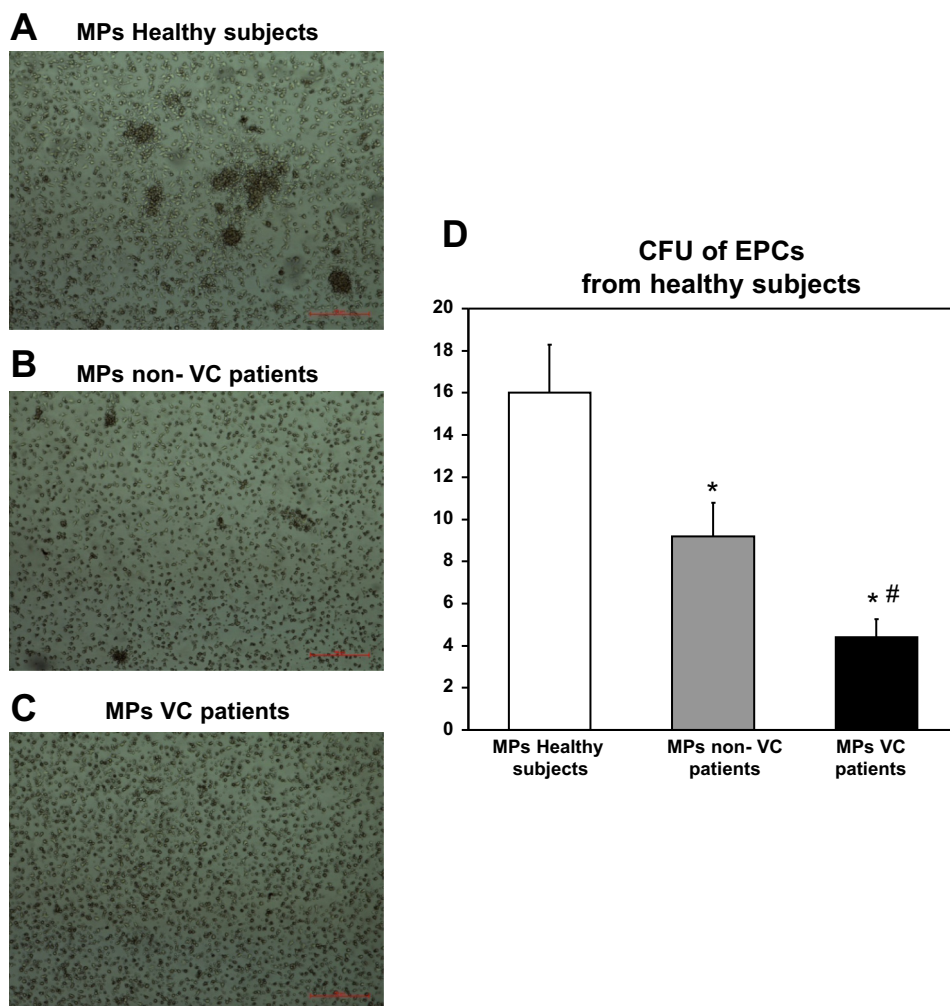
CKD patients with VC had a lower percentage of EPCs negative for CD34 than healthy subjects and non-VC patients (Table 3). We also noted a reduced number of CD34⁻EPCs in non-VC patients compared with healthy subjects. EPCs negative for CD133 were also quantified; the variation of these cells in CKD patients with and without VC and healthy subjects was similar to those observed in CD34⁻EPCs.

Expression of OCN in circulating EPCs. The expression of OCN in the subpopulations of EPCs was quantified. In healthy subjects, the number of EPCs that expressed OCN molecules was very low. The number of CD34⁻CD133⁺VEGFR2⁺EPCs that expressed OCN was higher in VC than non-VC patients, and both were significantly increased compared with

healthy subjects. Similarly, CKD patients displayed a significant increase in CD34⁺CD133⁺VEGFR2⁺EPCs that expressed OCN (*P* < 0.01 vs. healthy subjects), but the values for VC and non-VC patients were similar (Table 3). EPCs negative for CD133 and positive for OCN were elevated in CKD patients compared with healthy subjects.

Effect of CKD-derived MPs on OCN expression by EPCs and angiogenesis induced by EPCs. EPCs from healthy subjects were incubated for 1 wk with MPs from CKD patients with and without VC. MPs from VC and non-VC CKD patients inhibited the capacity of normal EPCs to form colonies (Fig. 3). Furthermore, MPs obtained from VC patients significantly reduced the number of colony-forming EPCs compared with those from non-VC patients (Fig. 3).

Fig. 3. Effect of microparticles (MPs) in EPC colony formation. EPCs obtained from healthy donors were cultured in a specific medium. A: representative images from the study of the capacity to form colonies when EPCs from healthy donors were cultivated with MPs from healthy subjects. B and C: EPCs from healthy donors were cultivated with MPs from non-VC patients (B) and from VC patients (C). D: quantification of colonies. **P* < 0.01 vs. MPs from healthy subjects; #*P* < 0.01 vs. MPs from non-VC patients. Results represent means ± SD of six independent experiments.



The *in vitro* effect of MPs on angiogenesis was also analyzed. EMP-obtained EPCs from healthy subjects were cultured for 4 h with MPs from CKD patients or control subjects. MPs from healthy subjects did not interfere with the formation of the tubular structures (Fig. 4A). However, EPCs cultured with MPs from CKD patients with VC were unable to form microtubules (Fig. 4C). MPs from non-VC patients also reduced the ability of EPCs to form tubes, but the effect was not as marked as with MPs from VC patients (Fig. 4B). The numbers of meshed areas (Fig. 4D) and Nb segments (Fig. 4E) decreased with MPs from patients with VC in relation to patients without VC. These parameters decreased with MPs from patients without VC compared with healthy subjects.

Incubation with MPs from CKD patients increased the expression of OCN by EPCs from healthy donors (Fig. 5, A–C). MPs from CKD patients with VC produced a greater increase in OCN expression than MPs from CKD patients without VC ($32 \pm 9\%$ in the VC group vs. $16 \pm 7\%$ in the non-VC group, $P < 0.01$; Fig. 5, B and C). This effect was observed in the three populations of EPCs evaluated: $CD34^-CD133^+VEGFR2^+$, $CD34^+CD133^+VEGFR2^+$, and $CD34^+CD133^-VEGFR2^+$ (Fig. 5, D–F). Using the same samples, the proportions of EPC subpopulations that expressed

OCN were calculated. MPs from VC patients produced an increase in the number of $CD34^-CD133^+VEGFR2^+OCN^+$ and $CD34^+CD133^+VEGFR2^+OCN^+$ EPCs compared with MPs of non-VC patients and healthy subjects (Fig. 5, D and E). The increase in OCN expression induced by MPs was particularly evident in $CD34^+CD133^-VEGFR2^+$ EPCs (Fig. 5F).

It was important to evaluate whether MPs from CKD patients were capable of inducing an osteogenic phenotype in monocytes and cells integrating the vascular wall. Incubation with MPs from CKD patients did not have an effect on OCN expression by endothelial cells and monocytes. In contrast, MPs from CKD patients with VC induced a threefold increase of OCN expression in VSMCs and also increased by more than twofold OCN expression in fibroblasts (Fig. 6). MPs from CKD patients without VC were also able to induce OCN expression in VSMCs, although the effect was not as marked as that observed with MPs from CKD patients with VC.

DISCUSSION

Our study shows that VC in CKD patients may be related to an alteration in the equilibrium between damage and repair of the endothelium. We have shown that OCN, a molecule implicated in the process of osteogenesis, is expressed in EPCs

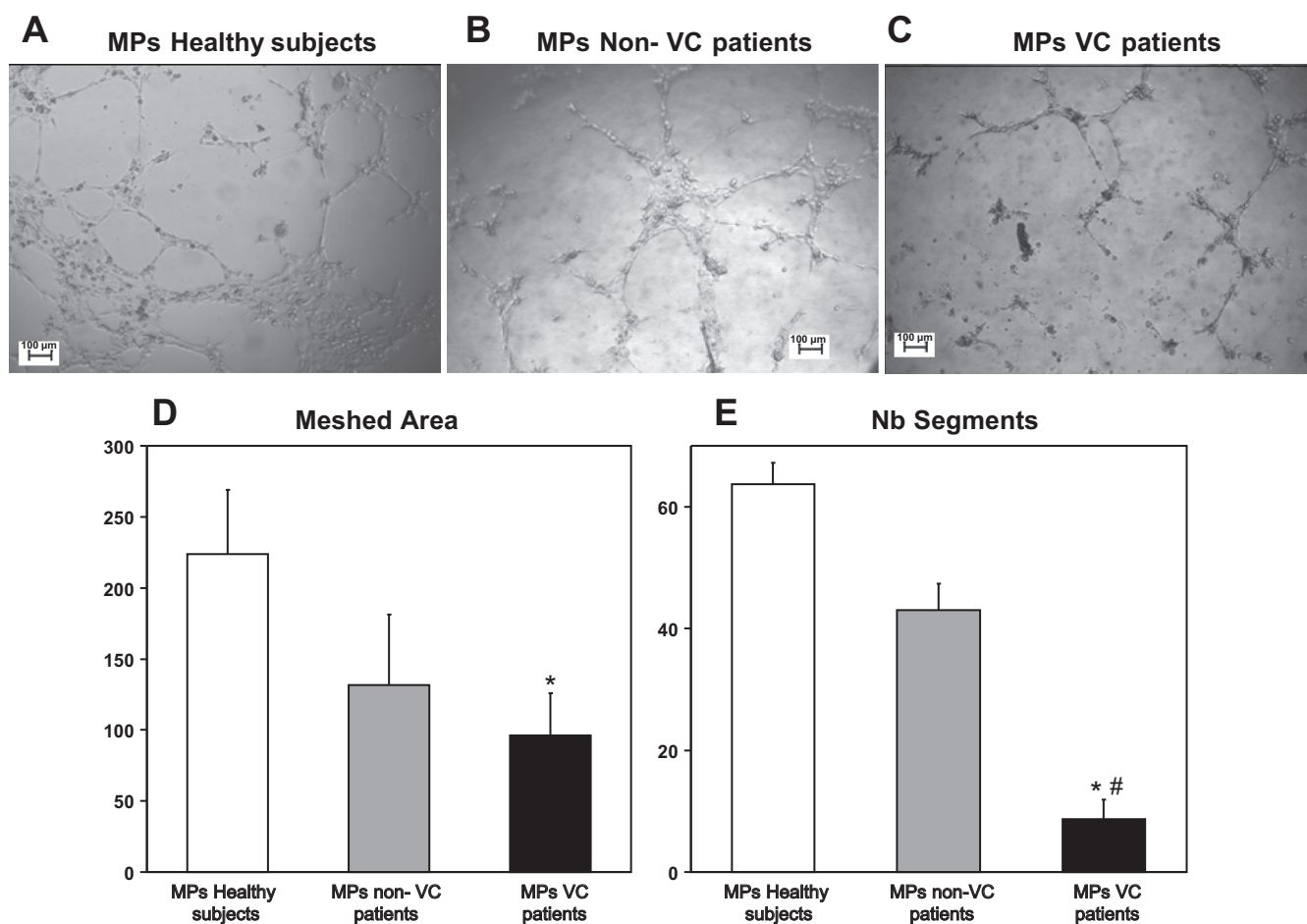


Fig. 4. Representative images of tube-like three-dimensional structures of EPCs in the presence of MPs. *A*: MPs from healthy subjects. *B*: MPs from non-VC patients. *C*: MPs from VC patients. *In vitro* angiogenesis was quantified by measuring the meshed area enclosed by segments or master segments (*D*) and the Nb segments or elements delimited by two junctions (*E*). Results represent means \pm SD of four independent experiments. * $P < 0.01$ vs. MPs from healthy subjects; # $P < 0.01$ vs. MPs from non-VC patients.

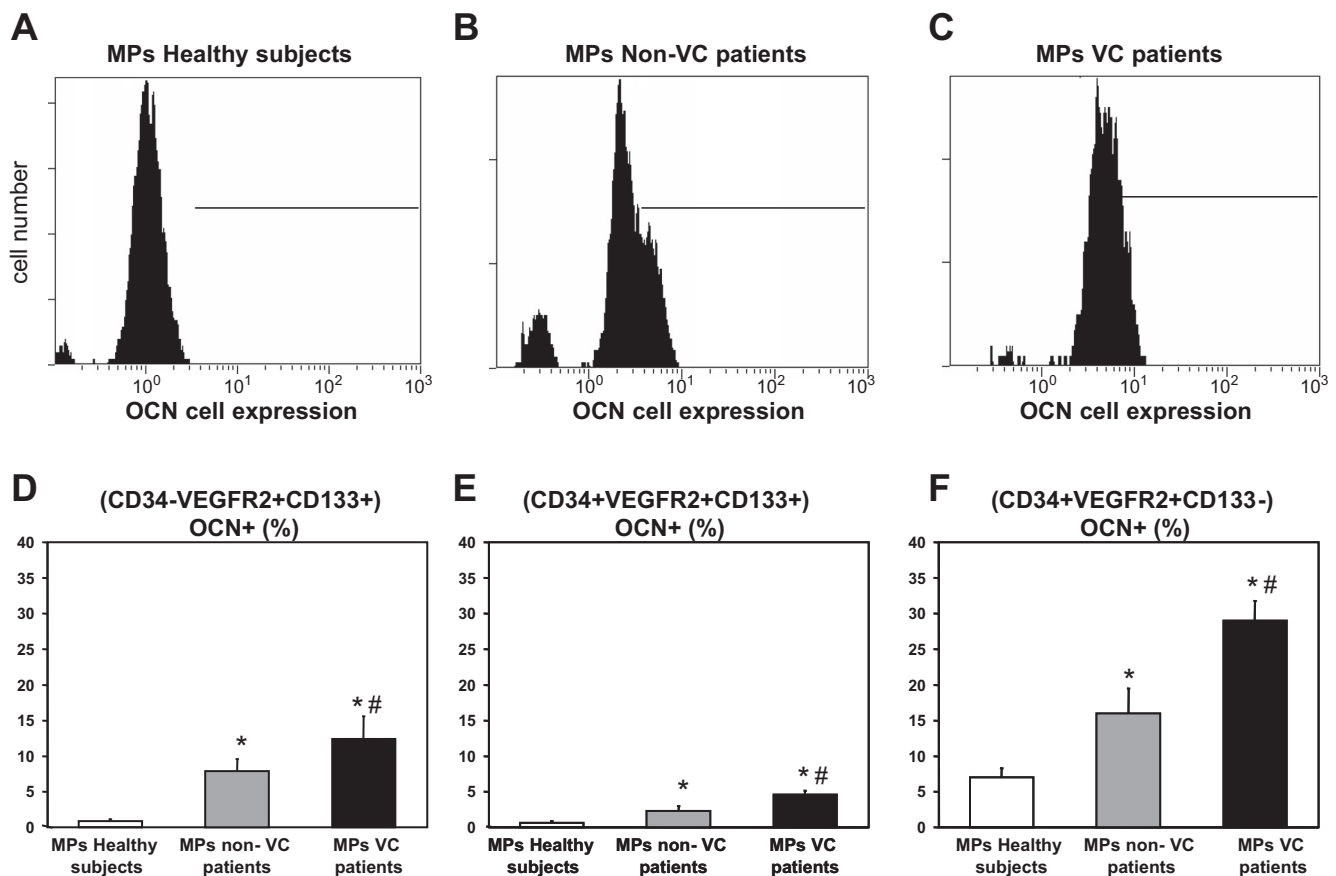


Fig. 5. Effects of MPs on osteocalcin (OCN) expression. EPCs obtained from healthy donors were cultured in a specific medium with MPs obtained from healthy subjects and VC and non-VC CKD patients. *A–C*: representative cytometric images of OCN expression. *D* and *E*: quantification of OCN expression in osteoblast EPCs. Results represent means \pm SD of six independent experiments. * $P < 0.01$ vs. MPs from healthy subjects; # $P < 0.01$ vs. MPs from non-VC patients.

from CKD patients with VC. In these patients, the number of EMPs is increased, and, in vitro, MPs from CKD patients with VC are capable to induce OCN expression in EPCs from healthy donors and in other cell lines (VSMCs and fibroblasts).

Although the therapeutic strategies available are relatively few and not very effective, we do not believe that VC is inevitable. VC was previously believed to be a passive degenerative process; presently, we know that the generation of VC is active and regulated, with a number of factors implicated (39). The identification of markers that will enable us to make a diagnosis and to assess the progression of VC would be a first step in the development of preventive and therapeutic tools to deal with the problem of VC in CKD patients.

In response to a stimulus or an injury, mature endothelial cells produce EMPs, which exhibit certain characteristics, such as the expression of molecules of endothelial origin like CD31 and phosphatidylserine, which bind to annexin V. We focused our attention on MPs of endothelial origin, assuming that the vascular damage associated to calcification had a pronounced effect on the endothelium; this does not mean that other cells involved in inflammation and/or tissue reparation may participate in the genesis of VC through the production of MPs. EMPs are very heterogeneous; the phenotype and functional properties vary according to factors involved in their release (22, 25). Moreover, an elevated number of EMPs reflects cellular injury as a surrogate marker of vascular dysfunction in

cardiovascular disease (40). In our study, CKD patients with VC had a greater number of EMPs than patients without VC.

Compared with healthy subjects, CKD patients either predialysis or on regular dialysis show an increase in EMPs. The number of EMPs in CKD patients may be modulated by treatment of the renal insufficiency (2, 31, 33). However, to the best of our knowledge, the present study shows, for the first time, an association between the increase of EMPs and the presence of VC in CKD patients.

The high prevalence of vascular damage in patients with CKD may also be associated with inadequate repair of the endothelium. Our findings are in agreement with previous studies (20, 34) showing that patients with CKD had a low number of EPCs and a reduced capacity to repair the endothelium. Moreover, the capacity to repair the endothelium was less in patients with VC than non-VC.

Several authors (19, 20) have found a relationship between the number of EPCs and cardiovascular morbidity/mortality. In fact, factors that are closely related to cardiovascular disease, such as arterial hypertension and high serum cholesterol levels, have been associated with a fall in the number of EPCs and potential regenerative capacity (36). Patients with CKD suffer from severe cardiovascular disease with high morbidity and mortality. Their survival may be influenced, at least in part, by a deficiency in vascular regeneration due to a reduction in the number of EPCs and/or in their functionality (2, 18, 24, 31,

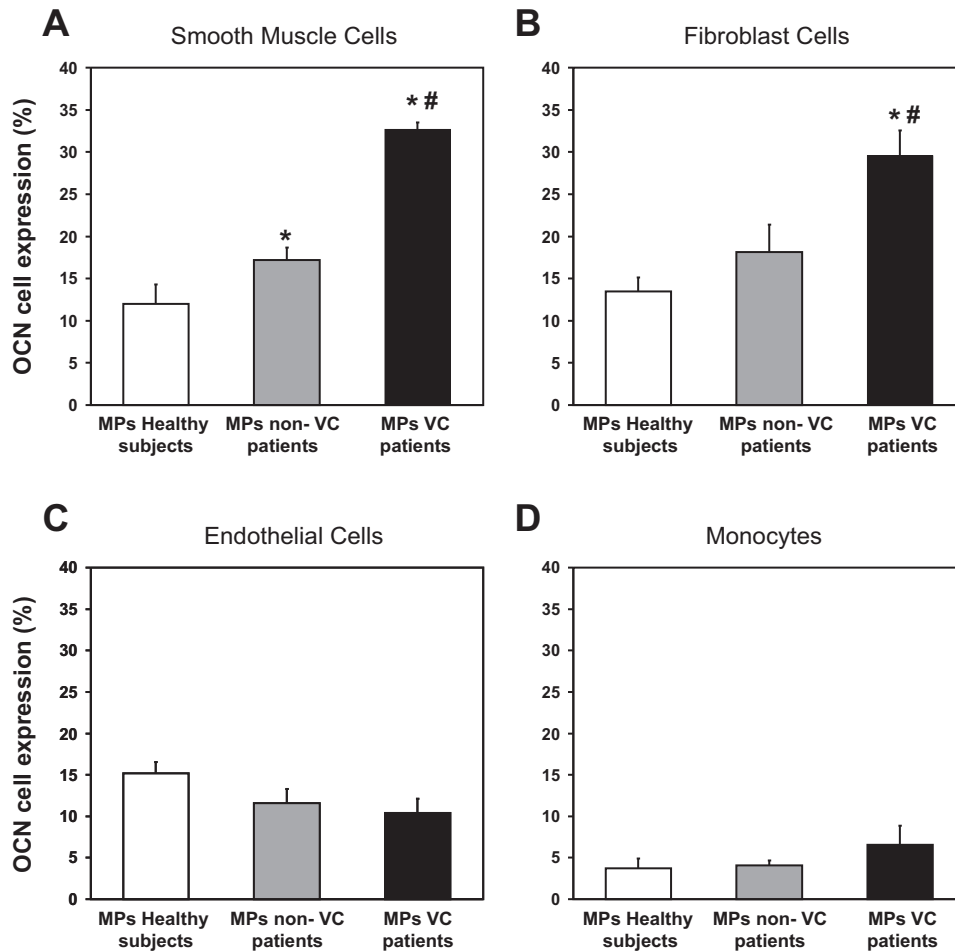


Fig. 6. Effects of MPs on OCN expression in cultured human cell lines. *A*: smooth muscle cells. *B*: fibroblast cells. *C*: mature endothelial cells (human umbilical vein endothelial cells). *D*: monocytes (THP-1 cells). * $P < 0.01$ vs. MPs from healthy subjects; # $P < 0.01$ vs. MPs from non-VC patients. Results represent means \pm SD of four independent experiments.

33). In our study, the proportion of hypertensive subjects was similar in both VC and non-VC patients. Cardiovascular events were more prevalent in patients in the VC group (42%) than in the non-VC group (22%); however, within each group, there was no significant association between the number and functional activity of EPCs or EMPs and cardiovascular events. CKD patients have numerous factors that predispose them to cardiovascular events; one important factor is VC. With our limited number of patients, it is difficult to demonstrate a separate association of VC and cardiovascular events with the number of EMP and the number and functional activity of EPCs.

We found that EPCs obtained from patients with VC may have osteogenic potential because they express OCN, which is a marker of osteoblastic cells. Other studies (11, 23, 28) have previously described the presence of osteoprogenitor markers both in the extracellular endothelial matrix and peripheral blood, since it appears to play an essential role in the repair and regeneration of bone. In CKD, the absolute number of EPC with proosteoblastic potential may be small, but they may have a functional impact considering that a relatively large proportion of these cells express OCN.

In patients with coronary atherosclerosis, there is a rise in the expression of OCN by EPCs (13, 16). Moreover, EPCs that express OCN have been found in greater numbers in calcified aortic valve tissue (17). In our study, we also obtained OCN⁺

EPCs when we cultured EPCs from healthy donors with MPs derived from CKD patients, a finding that reinforces the potentially dominant role played by MPs in the development of VC. Apart from EPCs, in vitro, MPs from CKD patients with VC had a remarkable capacity to induce OCN expression in VSMCs and fibroblasts.

The presence of precursor cells (OCN⁺ EPCs) that are common in peripheral blood may explain, at least in part, why “ossification” of the arteries may take place at any time and site. There may exist several stages in the process of endothelial repair. Endothelial damage results in the release of EMPs; these circulate in the peripheral blood and bone marrow, where they stimulate the production of EPCs required for endothelial repair. In normal subjects, physiological mechanisms between EMPs and EPCs maintain an equilibrium that is disrupted in the presence of diseases such as CKD, in which many uremic factors are involved. This repair mechanism is probably limited, and, at some point, the damage to the endothelium is much greater than the capacity of the bone marrow to produce EPCs, so alternative repair mechanisms are activated. In these circumstances, the organism use other progenitors originally designed to repair other types of tissue, such as bone, but end up repairing blood vessels, thus producing VC.

One limitation of the present study is the lack of information on the potential role of EMPs of origin other than endothelial cells, such as lymphocytes, endothelial cells, platelets, leuko-

cytes, and monocytes/macrophages. Further studies will have to be performed to define the role of other types of EMPs in the development of VC. We focused our attention on EMPs assuming that the vascular damage induced by calcification had a prevalent effect in endothelial cells. We are aware that the numbers of both EMPs and EPCs that we studied in peripheral blood were very low. To solve this technical problem, we measured a large number of events, with the aim of minimizing potential errors in the quantification. The number of patients in the study was limited. However, our results obtained from in vitro experiments were in agreement with the in vivo findings. Nevertheless, we believe that our experiments can help to understand some of the processes that taking place in the vasculature of patients with VC.

Our study has shown that in CKD patients with and without VC, there is an imbalance in the process of endothelial damage and repair, as suggested by the rise in EMPs paralleled with a decrease in the number of EPCs. The excess of MPs promotes the expression of an osteogenic phenotype in EPCs, VSMCs, and fibroblasts. We also show that EPCs may directly participate in the process of VC since some of these cells possess features of osteogenic cells. In fact, our findings can help to understand why patients with CKD frequently present VC. Our study also offers potential new clinical diagnostic tools, such as the increased number of apoptotic EMPs, low number of EPCs, and high percentage of cells with osteogenic characteristics. In conclusion, in CKD patients, the system of endothelial damage and repair may, through different mechanisms, participate in the generation of VC.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: S.S., F.T., M.R., M.A.-B., R.R., and J.C. conception and design of research; S.S., A.C., F.T., R.R., and J.C. analyzed data; S.S., A.C., F.T., M.R., R.R., P.A., and J.C. interpreted results of experiments; S.S., A.C., and J.C. prepared figures; S.S., A.C., F.T., A.M.-M., M.-A.A.-L., R.R., P.A., and J.C. drafted manuscript; S.S., M.R., M.A.-B., A.M.-M., M.-A.A.-L., R.R., P.A., and J.C. edited and revised manuscript; S.S., A.C., F.T., M.R., M.A.-B., A.M.-M., M.-A.A.-L., R.R., P.A., and J.C. approved final version of manuscript; A.C., F.T., and J.C. performed experiments.

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