Uremic medium causes expression, redistribution and shedding of adhesion molecules in cultured endothelial cells

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Background and Objectives. Patients with chronic renal failure show signs of accelerated atherosclerosis and high cardiovascular morbidity and mortality. Recent investigations indicate that uremia is associated with endothelial dysfunction and a microinflammatory state. We assessed changes in the expression of adhesion molecules [ELAM-1, VCAM-1 and ICAM-1], and proteins involved in hemostasis [von Willebrand factor (vWF) and thrombomodulin (TM)] in endothelial cells (ECs) and the corresponding extracellular matrices (ECM), respectively.

Design and Methods. Cultured human umbilical vein endothelial cells were incubated in the presence of a pool of normal or uremic sera. Immunocytochemical detection of related antigens was performed with specific antibodies coupled to colloidal gold. Concentrations of soluble adhesion molecules and TM in culture supernatants were evaluated by ELISA. Modifications in the transcription of the corresponding genes were also evaluated by Northern-blotting and reverse transcription polymerase chain reaction (RT-PCR).

Results. Exposure of ECs to uremic media caused an increase in the presence [ELAM-1, VCAM-1] and accessibility [ICAM-1] of the adhesion receptors on EC monolayers, as well as a higher presence of their soluble molecules in culture supernatants. We found a significant increase in the presence of vWF on the extracellular matrix derived from ECs grown in the presence of a uremic medium. The presence of TM on ECs and in the ECM remained unmodified, although there was a significant increase in the presence of TM in supernatants from ECs grown in the presence of uremic sera. Northern-blot or RT-PCR studies showed increased expression of the mRNA for all the corresponding genes (ELAM-1, VCAM-1, ICAM-1, vWF and TM).

Interpretation and Conclusions. Uremic medium causes inflammatory changes in ECs, which are characterized by enhanced expression, redistribution and shedding of adhesion molecules and TM, with an increased incorporation of vWF on the extracellular matrix generated.

Key words: uremia, endothelial cells, adhesion receptors, adhesive proteins, atherothrombosis.
ies suggest that hemodialysis procedures cause activation of platelets and leukocytes, which in turn may produce and release cytokines or other molecules with inflammatory potential. Mediators of inflammation, such as interleukin (IL) 1, TNF-α, and bacterial endotoxin, among others, act on endothelial cells to modulate two critical functional properties: inflammatory responses and the hemostatic process. Activation of endothelial cells is followed by modifications in the expression of adhesion molecules, such as ELAM-1, VCAM-1, and ICAM-1, thus providing regulatory mechanisms for leukocyte-vessel wall interactions. These mechanisms may be relevant in pathologic processes, such as acute and chronic progression of atherosclerosis. Studies suggest cellular dysfunction and inflammatory mechanisms concurrently acting on vascular endothelium, may contribute to the pathogenesis of atherosclerosis and to the increased rate of atherothombotic complications found in patients with chronic renal failure. Previous studies performed in the uremic setting by our group have provided information that supports these concepts.

Levels of a soluble circulating form of ELAM-1 (sELAM-1) have been reported to be increased in inflammatory disorders. Previous studies have demonstrated increased levels of soluble adhesion receptors, and other molecules derived from the endothelium in uremic patients. Although the clinical meaning of these observations is still unclear, the presence of soluble forms of adhesion molecules in circulating blood and increased levels of vWF and TM could represent markers of activation and/or damage at the endothelial level.

In the present study we investigated the effects of a uremic medium on the expression and redistribution of adhesion molecules in endothelial cells. For this purpose, endothelial cells harvested from human umbilical cords were initially grown in the presence of culture media containing normal serum. After reaching confluency, cells were exposed to growth media supplemented with normal or uremic serum for different periods of time. We analyzed i) the presence and distribution of adhesion receptors and TM on the surface of endothelial cells, ii) levels of soluble molecules in supernatants from the culture media, iii) modifications in proteins involved in hemostasis and expressed on the extracellular matrices, and iv) transcription of the corresponding genes, using Northern-blot or RT-PCR techniques.

### Design and Methods

**Experimental design**

Confluent endothelial cells (ECs) in culture were incubated with growth media containing uremic sera to evaluate: i) changes in the expression of ELAM-1, VCAM-1, ICAM-1 and TM in monolayers of ECs ii) levels of soluble adhesion molecules and TM in supernatants from the culture media, iii) modifications in the presence of vWF and TM on extracellular matrix generated by ECs (ECM), and iv) the expression of the mRNA for the adhesion receptors and vWF and TM genes. The presence of each antigen was detected by immunogold labeling using specific antibodies. Concentrations of soluble adhesion molecules in culture supernatants were evaluated by ELISA, and the expression of mRNA for the corresponding genes was analyzed by Northern-blot or RT-PCR.

**Human endothelial cell culture**

Endothelial cells were isolated from human umbilical cord veins according to a previously described method. Cells were maintained and subcultured with culture media (MEM 199; Gibco BRL, Life Technologies, Scotland) supplemented with 1 mM glutamine, 100 U/mL penicillin, 50 mg/mL streptomycin and 20% pooled human serum. When required, 50% of the human sera added to the growth media was from uremic patients. ECs were grown at 37ºC in a 5% CO₂ humidified incubator. The culture media were changed every 48 hours. After the second passage, ECs were subcultured on: a) 1% gelatin-coated glass coverslips, for immunogold labeling studies, and b) 50 cm² Roux flasks for RNA extraction.

**Culture of endothelial cells with serum from uremic patients**

The pool of sera from uremic patients was obtained from 10 patients with end-stage renal disease on maintenance hemodialysis. Five of the patients were men and 5 women; their mean age was 57±4.7 years, and their mean time on hemodialysis 80±20.9 months (mean±EEM). The cause of renal failure in these patients was: polycystic kidney disease (n=2), chronic glomerulonephritis (n=3), chronic pyelonephritis (n=2), nephrosclerosis (n=1), hemolytic uremic syndrome (n=1) and bilateral nephrectomy (n=1). All patients were dialysed for four hours three times a week with cellulose acetate membranes and bicarbonate-containing dialysate. The pool of control sera was obtained from healthy subjects. Informed consent to serum utilization was obtained from the patients.
ECs were incubated with growth media containing uremic sera for 24 hours, except otherwise indicated; when required, the ECMs from ECs, incubated with control or uremic medium, were exposed to 3% EGTA for 90 minutes.

Immunogold labeling and silver enhancement

Glass coverslips coated with either EC or ECM were fixed with 4% paraformaldehyde in 0.15M PBS, pH 7.4 (4°C, 10 min). Following extensive washing with PBS, fixed coverslips were incubated with the corresponding monoclonal antibodies (MoAb) against: ELAM-1 (CD36E) (Southern Biotechnology Associates, Inc., Birmingham, USA), VCAM-1 (CD106, clone P3C4), ICAM-1 (CD54, clone P2A4) (both from Progenetic, S.L., Barcelona, Spain), and thrombomodulin (CD 141) (American Diagnostica Inc.), and a polyclonal antibody against vWF (DAKO Diagnósticos, S.A., Barcelona, Spain)(dilution 1:100 for the MoAb and 1:250 for vWF Ab, 40 min, at room temperature).

After removing the excess of antibodies by washing (3X) with PBS, coverslips were incubated with a gold-conjugated goat anti-mouse IgG (Amersham Pharmacia Biotech) (dilution 1:2000, 40 min, at room temperature). The excess of antibody was removed by washing the coverslips with PBS (3X) and distilled water (3X). Finally, samples were treated with an intense silver enhancement reagent (Amersham Pharmacia Biotech). Samples were evaluated by light microscopy (Polyvar, Reichert-Jung, Wien, Austria) and the density of labeling was calculated by computerized morphometric analysis (SigmaScan Pro, Jandel Scientific, Germany).

Detection of soluble adhesion molecules and thrombomodulin

sELAM-1, sVCAM-1, sICAM-1 and TM concentrations in control or uremic cell culture supernatants were measured by colorimetric-Sandwich ELISA kits (Parameter human sE-selectin, Parameter human sVCAM-1 and Parameter human ICAM-1, R&D Systems, Abingdon, United Kingdom, and IMUBIND Thrombomodulin ELISA kit, American Diagnostica Inc., Greenwich, United Kingdom). The supernatants were obtained from ECs cultured with control or uremic media for 24 hours. Prior to the assay, supernatants were centrifuged for 5 minutes at 1,100 rpm to remove non-adherent cells and stored at -20°C. Color absorbance was read at 630nm for the soluble adhesion receptors, and at 450nm for thrombomodulin.

Northern-blot and reverse transcription-polymerase chain reaction analysis of mRNA endothelial cells

After reaching confluency, ECs were incubated with uremic medium for 4, 16, 24 and 48 hours to detect changes in the expression of mRNA for the adhesion receptors. In the case of mRNA for TM and vWF, ECs were incubated for only 24 hours. Total RNA was extracted from 5-10×10⁶ ECs using TriPure® solution. For Northern-blot studies, each DNA probe was radiolabeled using a RandonPrimer labeling system ( Gibco BRL, Life Technologies, Scotland). β-actin probe was obtained by PCR, using primers from R&D Systems (Abingdon, United Kingdom); TM cDNA was purchased from ATCC (USA), cloned in pUC19; the vWF probe was a gift from Dr. P. Tjernberg and Dr. J. Eikenboom (Trombose en Hemostase Research Centrum, Leiden, The Netherlands). For RT-PCR, 10µg total RNA from each sample were denatured at 70°C for 5 min and chilled rapidly on ice. The RNA was then reverse transcribed using the Superscript™ RNase H- Reverse transcriptase kit (Invitrogen, Life Technologies, Scotland). After the reverse transcription, PCR amplification was performed. To check that equal amounts of RNA were reverse-transcribed, the same RNA was amplified with β-actin primers. ICAM-1, ELAM-1 and β-actin primers employed were from R&D Systems (Abingdon, United Kingdom). The sequence of VCAM-1 primers was as follows: 5HTF: 5' GCTCTGTGACCATGACCTGTTC 3' and 3HTF: 5'CTGACCAAGACWTTGTATCTC 3'24 Each PCR product was electrophoresed through a 1% agarose gel, stained with ethidium bromide, and photographed under UV transillumination.

Statistical analysis

Results from the experiments are expressed as mean ± standard error of the mean. Statistical differences were analyzed using Student's t test for paired data (Primer of Biostatistics; McGraw-Hill). A p<0.05 was considered statistically significant.

Results

Adhesion molecules in endothelial cell monolayers and in cultured media supernatants

Microscopy evaluation revealed an increased presence of ELAM-1, VCAM-1 and ICAM-1 receptors at sites of intercellular contacts in those ECs exposed to uremic medium for 24 hours. Figure 1
shows micrographs illustrating expression of ELAM-1, VCAM-1 and ICAM-1 on EC monolayers incubated with normal (A, B, C) or uremic medium (D, E, F) for 24 hours. Cells were incubated with the corresponding monoclonal antibodies to detect ELAM-1 (A, D), VCAM-1 (B, E) and ICAM-1 (C, F), followed by immunogold and silver enhancement techniques. An increased presence of immunogold labeling related to the different adhesion receptors was observed mainly located at the intercellular contacts of endothelial cells exposed to uremic media. A and B some gold particles bound to the antigen were initially detected. Images are representative of 5 different experiments (X320). Bar equals 20 µm.

Results are summarized in Figure 2 and revealed a statistically significant increase in the presence of all soluble antigens screened when ECs were incubated with uremic medium for 24 hours. Average values for sELAM-1 reached levels of 16.8±0.9 ng/mL after exposure to uremic medium (p<0.01 vs. 11.8±0.8 ng/mL in the respective paired control, n=9). Similarly, levels of sVCAM-1 and sICAM-1 reached levels of 48.7±2.6 and 137.0±13.5 ng/mL, n=11, when ECs had been exposed to uremic medium (p<0.01 vs. 36.4±2.0 ng/mL and 86.4±9.5 ng/mL in their respective paired controls, n=8).

Detection of von Willebrand factor and thrombomodulin on endothelial cells

After immunocytochemical detection, the density of labeling (expressed in arbitrary units obtained by densitometric analysis) for vWF was higher on
ECMs derived from ECs exposed to uremic medium for 24 hours (10.2±1.4 vs. 5.6±0.6 in their corresponding matched controls, p<0.05, n=5). The presence of TM remained unmodified both on the ECM (3.0±1.4 vs. 3.7±1.7 in controls ECMs, p=0.135, n=5) and at the membrane of ECs incubated with uremic media for 24 hours (2.95±1.2 vs. 3.11±1.33, p=0.185, n=5). Micrographs in Figure 3 illustrate these results.

Soluble thrombomodulin in control and uremic culture supernatants was also measured, this protein showing a higher presence in supernatants from ECs incubated with uremic medium for 24 hours (1.1±0.1 vs. 0.3±0.0 ng/mL in the respective paired control, p<0.01, n=11).

Sequential studies on mRNA expression for adhesion receptors and proteins involved in hemostasis

Under standard culture conditions ECs express baseline levels of ICAM-1 but lack ELAM-1 and VCAM-1 expression. Exposure to uremic media for 4, 16-24 and 48 hours revealed sequential changes in the expression of the corresponding genes. Expression of ELAM-1 peaked after 4 hours of incubation with uremic media and then tended to decrease. VCAM-1 mRNA expression peaked after 4 hours of incubation and decreased progressively from 16 to 24 hours, almost disappearing by 48 hours. ICAM-1 gene was upregulated when ECs were incubated with uremic media. The major expression for this gene was observed at 4 hours and it then decreased between 16 and 24 hours. The modifications described were never observed when ECs were exposed to control serum. Results of these studies are summarized in Figure 4.

A weak signal was observed in lanes corresponding to mRNA from control ECs. Incubation of ECs with culture media supplemented with 50% uremic serum for 24 hours resulted in increased expression of mRNA for both vWF and TM. These results are illustrated in Figure 4b.

Discussion

In vitro exposure of ECs to uremic media results in an increased expression and redistribution of adhesion molecules on EC monolayers, with enhanced shedding of adhesion molecules and soluble TM into culture supernatants. An increased presence of vWF, an indirect marker of endothelial dysfunction, was also evident on the extracellular matrices generated by ECs. These experimental observations confirm that the uremic medium is capable of inducing a pro-inflammatory state on cultured human endothelial cells, which indicates its contribution to the development and/or acceleration of atherosclerosis described in this group of patients.

The greater incidence of cardiovascular morbidity and mortality present in this group of patients suggests the existence of a prothrombotic and proatherogenic state. ECs covering the inner vascular layer are now considered an interface playing a
critical role in the regulation of vascular tone and growth, in the recruitment and transmigration of leukocytes into the site of inflammation or tissue injury, in the maintenance of a balanced hemostasis and in the composition of the extracellular matrix. Dysfunction of the vascular endothelium is considered a critical event for the initiation of atherosclerosis.

The factors postulated to be involved in the endothelial dysfunction of uremia include: inflammation, retention of L-arginine inhibitors (such as ADMA, SDMA), oxidative stress, hyperhomocysteinemia, advanced glycosylation end-products (AGEs), or oxidized lipoproteins (ALEs), among others. Moreover, the hemodialysis procedure per se induces intense activation of blood cell elements with subsequent cytokine release, which may aggravate the inflammatory state and endothelial dysfunction. In this regard, increased levels of pro-inflammatory cytokines, such as IL-1, IL-6 and TNF-γ, and C-reactive protein have been reported in dialysis patients. The increased expression of ELAM-1, VCAM-1 and ICAM-1 on activated endothelium is thought to represent a mechanism through which various leukocyte subtypes can be rapidly recruited into sites of inflammation or tissue injury. Results of our experimental studies demonstrate that the uremic medium enhances the presence of these proteins at the sites of cell-to-cell interactions. Moreover, our findings correlated with an increased expression of the genes for ELAM-1, VCAM-1 and ICAM-1 in ECs exposed to uremic media for 4 hours. These results suggest that exposure to uremic media leads to a pro-inflammatory state that affects the vascular endothelium.

Expression of adhesion receptors may be induced on the cell surface of both vascular endothelial cells and leukocytes, among other cells, under certain pathologic situations. Increased levels of these molecules have been found in infective and inflammatory processes. In this regard, it has been recently reported that hemodialysis patients present elevated serum levels of certain adhesion molecules and other markers of endothelial dysfunction. In agreement with these in vivo studies, our findings indicate that exposure of endothelial cells to uremic media results in increased levels of sELAM-1, sVCAM-1 and sICAM-1 in culture supernatants. Release of soluble adhesion molecules from the cell surface has been proposed as a mechanism for down-regulating cytokine-induced increases in receptor expression. This event may be a prerequisite for the cell capture phase of adhesion to cell extravasation. Soluble molecules may function as competitive inhibitors of membrane bound forms, thereby regulating cell adhesion.

Our present results showing a higher expression of adhesion receptors at the cell membrane and also of the corresponding soluble forms were correlated with increases in expression of the corre-
sponding mRNA. ECs exhibit de novo expression of ELAM-1 and VCAM-1, and upregulation of ICAM-1, only after activation by cytokines or other mediators of inflammation, such as IL-1 and TNF-1. The increased expression of mRNA for these molecules found in the present study follows the same pattern as that observed in ECs exposed to pro-inflammatory cytokines, thus suggesting that the effect of uremic media on ECs could be mediated, at least in part, by pro-inflammatory cytokines described to be increased in uremia.

Through the present study, we have found changes in the levels of proteins involved in hemostasis, such as vWF and TM. vWF is synthesized by ECs and stored in Weibel-Palade bodies. When released into plasma and the ECM, vWF mediates platelet adhesion and aggregation to vascular subendothelium. vWF has been proposed as a marker of endothelial dysfunction since its release is increased when ECs are exposed to damaging conditions. Increased levels of circulating vWF have been described in uremic patients. These abnormally high levels of plasma vWF have been found to be correlated with low values of the protease for vWF. Surprisingly, the adhesive functions of the vWF present in plasma from uremic patients seem to be markedly deficient.

In the present study, the presence of vWF was augmented on the ECMs generated by ECs cultured in the presence of uremic sera. Moreover, this enhanced expression of vWF was associated with an increase in mRNA transcription by the corresponding gene. This observation would further support the concept that exposure of ECs to uremic medium causes an activating state in endothelial cells as derived by the increased release of vWF. In a previous study, our group demonstrated that the composition of the ECM generated by endothelial cells exposed to uremic medium for a prolonged period of time is altered.

This alteration was evidenced by a decreased reactivity towards platelets when the ECMs were exposed to circulating blood anticoagulated with citrate. Results of the present study reveal that the decreased reactivity found in studies in which coagulation mechanisms were inhibited could be due to qualitative alterations in the functional properties of the vWF produced in this clinical condition. These functional alterations would counterbalance the quantitative increases of this adhesive protein observed in uremic patients.

On the other hand, we failed to detect modifications of TM at the cell membrane, despite the fact that a clear increase in the expression of mRNA for TM was observed in cells exposed to a uremic medium. This increase was correlated with a post-transcriptional expression of soluble TM. TM is a membrane glycoprotein mostly located on endothelial cells, although small amounts appear in the circulation in a soluble form. Its pathophysiologic function is not fully understood, but it is thought to represent a marker of endothelial cell damage. In this regard, it has been recently reported that hemodialysed patients present higher serum levels of thrombomodulin than do healthy subjects. Our results confirm this previous evidence, reinforcing the occurrence of an ongoing stress on the endothelium in uremia.

In conclusion, the results obtained in the present study indicate that uremic media alter critical functional aspects of endothelial cells in vitro, and may alter the hemostatic balance of the vascular endothelial wall in patients with end-stage renal disease. Uremic patients suffer from complex alterations of hemostasis and are known to develop accelerated atherosclerosis.

Our present data reinforce the concept that uremic media contribute to the development of endothelial cell dysfunction that would induce a pro-inflammatory and a prothrombotic state, eventually leading to accelerated atherosclerosis and enhanced thrombosis.

Contributions and Acknowledgments
MS was the principal investigator primarily responsible for the manuscript writing and for all the figures. She carried out the experimental part of the study, and participated in its design, and the analysis and interpretation of the results. MDR was responsible for the conception of the study and for its critical revision. MJZ helped with the experimental part. AC contributed to the design and critical revision of the study. We thank AO for his contribution to the conception of the discussion and critical revision of the study. GE was responsible for handling and interpreting the data and direct supervision. MS, MDR and GE wrote the manuscript. All the authors approved the version submitted.

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**PEER REVIEW OUTCOMES**

**Manuscript processing**

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**What is already known on this topic**

Patients with chronic renal failure, independently of dialysis treatment, have with mild, chronic inflammation, endothelial dysfunction and hemostatic activation. The initial event(s) triggering these processes is still unknown.

**What this study adds**

This study shows that a pool of uremic sera obtained from patients on hemodialysis contains some factor(s) not yet identified which induce the synthesis, secretion and shedding of several inflammatory and hemostatic proteins by cultured HUVEC. Thus, some of the characteristics observed in uremic endothelial cells in vivo appear to be reproduced by the experimental conditions designed in this study.

**Potential implications for clinical practice**

We still need to identify the initial inflammatory trigger and the factor(s) mediating these effects on EC and how this dysfunctional endothelium contributes to the accelerated atherosclerotic process in patients with chronic renal failure.

Diego Mezzano, Associate Editor (Santiago, Chile)