ORIGINAL RESEARCH

Haemodialysis through a cellulose membrane induces dephosphorylation of CD11b and promotes leukocyte adhesion to endothelial cells

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Abstract

Purpose: To explore modifications in signal mechanisms involving CD11b and leukocyte adhesion in patients under haemodialysis (HD).

Methods: Samples were obtained from uremic patients at baseline, 15 and 120 min of HD from both arterial and venous lines. CD11b expression was studied by flow cytometry. To study signalling mechanisms, CD11b was immunoprecipitated using a specific antibody. Immunoprecipitates were resolved by 8% SDS-PAGE to measure phosphorylation in immunoblots. Leukocyte adhesion was measured after blood perfusion using endothelial cells (EC) as adhesive substrate. Parallel studies were performed with blood from healthy donors.

Results: The percentage of CD11b⁺ cells increased during HD with a cellulose membrane in the venous line at 15 and 120 min ($6.2 \pm 2.9\%$ and $11.0 \pm 7.1\%$) and in the arterial line at 120 min (11.5 ± 8.5 vs. $3.1 \pm 1.0\%$ in control $P < 0.05$). After 120 min HD, CD11b phosphorylation decreased in leukocytes from both arterial (72.6 ± 2.9) and venous lines (51.8 ± 6.5) vs. basal samples (119.5 ± 15.5 $P < 0.005$). Control leukocytes showed enhanced adhesion to uremic EC compared with control EC (3.0 ± 0.3 vs. 2.3 ± 1.0 leukocytes $\times 100$ EC⁻¹ $P < 0.05$). Uremic leukocyte adhesion was enhanced after HD compared with basal samples 4.2 ± 0.2 leukocytes/100 EC in the arterial and 4.4 ± 0.3 in the venous line; after 120 min vs 2.3 ± 1.0 ($P < 0.005$).

Conclusion: Leukocyte activation during HD through a cellulose membrane occurs with decreases in CD11b phosphorylation. Activation also induces increases in CD11b expression associated with enhanced leukocyte adhesion to uremic endothelial cells.

Patients with end-stage renal disease (ESRD) suffer from complex hemostatic disorders. Inflammation is a common finding in patients with advanced kidney disease. About 30-50% of dialyzed patients exhibit a chronic inflammatory state associated with impaired polymorphonuclear neutrophil function. This results in a decrement in endothelial function accompanied by an acceleration of atherosclerosis and an increase of cardiovascular risk.¹⁻⁴ Recognition of the scope and extent of this problem in ESRD patients has led to efforts to develop new therapeutic approaches designed to prevent cardiovascular morbidity and mortality in uremic patients.

Migration of leukocytes from the peripheral circulation into interstitial spaces involves sequential interactions between activation-dependent molecules on the endothelium and leukocytes. The array of physio-

logical responses associated with adhesion molecules is coordinated by the CD11b/CD18 complex.^{5,6} CD11b promotes adhesion of leukocytes to endothelial cells through Inter Cellular Adhesion Molecule (ICAM) and Endothelial Leukocyte Adhesion Molecule (ELAM) interactions and mediates the transendothelial migration of circulating neutrophils in the early stages of the inflammation.³ The affinity or avidity of integrins for their extracellular ligands can change in response to cytoplasmic signals initiated by the stimulation of cellular receptors.^{7,8} This 'activation' of integrins has been termed 'inside-out' signalling. Integrins also activate intracellular signalling pathways in a process termed 'outside-in' signalling and enhanced cell adhesion.⁹⁻¹² The balance between phosphorylation and dephosphorylation of integrins might be a mechanism by which both integrin activation and signal transduction could be regulated.^{13,14} Phosphorylation events are believed to mediate 'outside-in' signal transduction by integrins and are generally induced by cell adhesion.¹³ In studies on integrin phosphorylation, the α chain was shown to be constitutively phosphorylated, whereas the β_2 chain was reported to become phosphorylated only after activation.^{15,16} Phosphoamino acid analysis of CD11b/18 reveals available serine, threonine, and tyrosine that may serve as putative phosphorylation sites for specific protein kinases.¹⁶

In previous studies we have demonstrated leukocyte activation in uremic patients.^{2,17} In the present study, we have explored the relationship between expression of CD11b, leukocyte adhesion and the phosphorylation status of tyrosine, serine and threonine residues of CD11b in leukocytes from uremic patients at baseline and throughout haemodialysis with a cellulose membrane.

Subjects and methods

The study was approved by the Ethical Committee of the Hospital, and was performed according to the Dec-

laration of Helsinki. Informed consent was obtained from all participants.

Patients

Samples from ten patients with end-stage renal disease (eight men and two women; mean age 51.3 yr, range 34-75 yr) undergoing maintenance haemodialysis treatment for 4 hr, 3 times a week, were evaluated. The causes of renal failure were glomerulonephritis (n=2), chronic pyelonephritis (n=2), uremic hemolytic syndrome (n=1), diabetes (n=1), polycystic kidney disease (n=1), analgesic nephropathy (n=1), lupus nephritis (n=1), and bilateral nephrectomy (n=1). Nine of the patients had been receiving recombinant human erythropoietin (ranging from 6000 to 9000 IU / week), for the treatment of anaemia for at least 6 months prior to the study. Samples from ten healthy subjects with similar characteristics were included in the study as a control group.

Study design

All patients had been on haemodialysis in our hospital for at least 12 months with ethylene oxide sterilized cellulose acetate-based membranes (MN-140, surface area 1.4 m² or MN-170 surface area 1.7 m², Althin Medical, Miami Lakes, FL). A bicarbonate-based dialysate was used in all cases. Samples were drawn before dialysis from the arterial and venous lines at 15 and 120 min and anticoagulated with citrate (3.8%, w/v). Leukocyte activation was measured by analysing modifications both in CD11b expression and in CD11b phosphorylation as well as changes in leukocyte adhesive behaviour.

Isolation of leukocytes

Leukocytes were prepared according to a previously described method with minor modifications.¹⁸ Briefly, after 60 min sedimentation with 10% dextran (vol/vol) (Sigma Chemical, St. Louis, MO, USA), the resulting leukocyte suspension was exposed to an osmotic shock to eliminate contaminating erythrocytes. The red cell-

free fraction was then washed 3 times by centrifugation (1,000 g, 10 min, 4°C) in Hanks' HEPEs (HH) buffer, pH 7.2, to which 5 mg/ml of bovine albumin (Sigma) was added. Potentially contaminating platelets were checked for each experiment and the ratio leukocytes: platelets was never observed to be >1:10.

Expression of CD11b.

Expression of leukocyte integrin CD11b was analyzed by flow cytometry. Immunolabeling of leukocytes with monoclonal antibodies (MoAbs) was performed in whole blood. Briefly, after collection and fixation, samples were diluted by adding 5 µL of whole blood to polypropylene tubes preloaded with 50 µL phosphate buffered saline (PBS) (Bio Mérieux, Marcy l'Etoile, France). Samples were incubated, for 20 min, in the dark, at room temperature, with 10 µL of monoclonal antibodies ICRF44 fluorescein isothiocyanate (FITC)-conjugated against CD11b and B-A11 phycoerythrin (PE) against CD45 or PE isotypic antibody to determine "non specific background" and instrument noise. MoAbs were purchased from Serotec (Oxford, England). Samples were then diluted with 1 mL of PBS and analyzed with a FACScan flow cytometer (Becton-Dickinson, Mountain View, Ca). Fluorescence and scatter signals were calibrated with 2 µm calibrate beads (Becton-Dickinson.). Five thousand cells were analyzed in each sample. All values were subtracted from those obtained with the control antibody.

Percentage of positive cells was used to evaluate antibody binding. To study the percentage of positive cells, an analytical marker was set in the green fluorescence channel to define 2% of the cell population with the highest membrane fluorescence at the baseline level. This marker was used as a threshold to determine the proportion of cells exhibiting immunofluorescence above this level in all subsequent samples.

Phosphorylation of CD11b in leukocytes

Leukocytes (1×10^7) were lysed with RIPA buffer (50 mM TRIS, 150 mM Na Cl, 1% IGEPAL, 0.25 mM Deoxycholate, 1 mM EGTA) containing 1mM sodium orthovanadate, 1 mM PMSF, 1mM NaF 1 µg/ml leupeptin and 1 µg/ml pepstatin, as protease and phosphatase inhibitors. Lysed cells were sonicated during 15 sec and centrifuged at 14.000 g. Supernatants were incubated with anti-CD11b from Chemicon International (Temecula, CA, USA), overnight at 4°C. Then, samples were precleared by incubation with 100 µL of protein A/beaded agarose (Pierce Rockford IL USA) and rabbit IgG (Dako Denmark) for 1h at 4°C. Beads were pelleted and washed with RIPA buffer. Immunoprecipitates were solubilized with Laemmli's buffer (125 mM Tris-HCl, 2% SDS, 5% glycerol and 0.003% bromophenol blue) containing inhibitors as well. After 15 minutes at 4°C, lysates were collected in an Eppendorf, and heated at 90°C for 5 minutes. Samples were resolved by 8% SDS-polyacrylamide gels and proteins transferred to nitrocellulose membrane.¹⁹ Electrophoresis and immunoblot reagents were from Bio-RAD Laboratories S.A. (Madrid, Spain).

Membranes to detect phosphorylated forms of CD11b were proved with a Rabbit antibody to phosphoS/T/Y conjugated to horseradish peroxidase (Zymed Laboratories Inc San Francisco CA USA) and developed by the enhanced chemiluminescence (ECL) method²⁰ (reagents, Hyperfilm-ECL). Protein bands were densitometrically analysed (Kodak Digital Science 1D, Eastman Kodak Company, Rochester, New York). Presence of CD11b was confirmed using the CD11b antibody. A rabbit antibody specific to PKC (Santa Cruz Biotechnology Santa Cruz CA USA) was also used in some experiments

Endothelial Cell Culture

Endothelial cells (EC) obtained from human umbilical cord veins were cultured according to the method described by Jaffe *et al*²¹ with minor modifications.

Cells were maintained at 37°C in a 5% CO₂ humidified incubator and subcultured with culture medium (MEM 199; Gibco BRL, Life Technologies, Scotland) supplemented with 1 mM glutamine, 100 U/mL penicillin, 50 µg/mL streptomycin and 20% pooled human serum (control EC). In some experiments, EC were grown in the presence of serum from uremic patients (50% of total sera) to reproduce the endothelial dysfunction model (uremic EC)²². The culture medium was changed every 48 h. After the second passage, EC were subcultured on 1% gelatin precoated glass coverslips for perfusion studies.

Adhesion of leukocytes under flow conditions

Perfusion studies were carried out in a parallel-plate chamber according to the method described by Sakariassen.²³ Blood was recirculated through the chamber (10 min, shear rate of 450 sec⁻¹), using a peristaltic pump. Two coverslips were inserted into the chamber covered with a confluent EC monolayer. At the end of the perfusion, the coverslips were removed from the chamber, rinsed with 0.15 M phosphate buffered saline (PBS) and stained with May Grunwald-Giemsa.

Count of leukocyte adhered to EC

Analysis of stained coverslips was performed using a light microscope connected to an image analyser through a video camera. Adherent cells were counted by digital image processing. We quantified the number of leukocytes and endothelial cells in a standardized area (1.3x10⁷ pixels). Five standardized areas (EC average number of 500) were analysed for each experiment and 3 independent blinded investigators analysed samples. Results were expressed as leukocytes/100 EC.

Data analysis

Results were expressed as mean ± SEM. Statistical analyses were performed by means of one way analysis of variance (ANOVA) to compare differences be-

tween controls, and dialyzed patients. Repeated measures ANOVA were used to compare the changes of the parameters throughout the haemodialysis session. The correlation coefficient was used to indicate the relationship between CD11b expression and leukocyte adhesion. Differences between groups were considered significant if $P < 0.05$.

Results

Evaluation of CD11b expression

Percentages of CD11b positive cells in samples from uremic patients before hemodialysis with a cellulose membrane showed a non significant decrease with respect to healthy subjects. Cellulose membranes increased the percentages of CD11b positive cells throughout the hemodialysis. Differences reached statistical significance at 15 min and 120 min in venous line (6.2±2.9 and 11.0±2.7 respectively) and at 120 min in arterial line (11.5±3.3) vs. baseline values 3.1±1.0; $P < 0.05$). (Fig 1).

Changes in the phosphorylation levels of CD11b during haemodialysis

CD11b was constitutively phosphorylated in uremic patients under basal conditions (Fig 2) showing values of phosphorylation intensity similar to those observed in healthy donors (119.5±15.5 vs. 111.3±19.6,

FIGURE 1. Up regulation of CD11b in leukocytes induced by a cellulose membrane. Results are expressed as % of cells CD11b+ (Mean ±S.E.M).

FIGURE 2. Immunoprecipitation of the CD11b in leukocytes from uremic patients under dialysis with a cellulose membrane. Panel A - kinetics of serine, threonine and tyrosine phosphorylation in immunoprecipitates of CD11b, which results in the co-precipitation of a 75 kD protein. Panel B - presence of the CD11b was confirmed. The images are from 1 representative experiment out of 10.

On the right bar diagram summarizing changes in CD11b and a 75 kD protein phosphorylation. Results are expressed as mean intensity of phosphorylation (Mean $\pm$ S.E.M, n=10, * $p < 0.05$ ** $p < 0.005$).

Control, Pre: predialysis, A: arterial line, V: venous line, IP: immunoprecipitation and IB: immunoblot with an anti-phosphoserine, anti-phosphothreonine, anti-phosphotyrosine conjugated with horseradish peroxidase.

Mean $\pm$ SD, n=10). After 120 min haemodialysis, phosphorylation of CD11b exhibited a decrease in blood obtained from both arterial and venous lines (phosphorylation intensity of 72.6 ± 2.9 , $P < 0.05$ and 51.8 ± 6.5 , $P < 0.005$) respectively, compared with basal levels (119.5 ± 15.5). (Fig 2) Phosphorylation in samples obtained after 15 min haemodialysis remained unchanged. There were no changes in CD18, the β subunit associated with CD11b. A 75 kD protein

showed dephosphorylation after 120 min of hemodialysis (figure 2) Although it was initially suspected to be PKC, immunoblots were probed with a specific antibody and did not provide confirmation (data not shown).

Adhesion of leukocytes on endothelial cells

Adhesion experiments were performed using blood from healthy donors and uremic patients perfused on

FIGURE 3. Bar diagram summarizing changes of leukocyte adhesion to endothelial cell. Number of leukocytes/100 endothelial cells. (Mean $\pm$ SEM; n=10, * $P < 0.05$). Light micrographs representative of leukocyte adhesion to uremic endothelial cell.

endothelial cells cultured with medium containing control sera (control EC) or uremic sera (uremic EC). Four different settings were carried out: (i) perfusions of control blood on control EC; (ii) perfusions of control blood on uremic EC; (iii) perfusions of uremic blood on control EC; and (iv) perfusions of uremic blood on uremic EC.

In adhesion studies using control blood samples, we observed higher levels of leukocyte adhesion on uremic EC (3.0 ± 0.3 leukocytes/100 EC) than on control EC (2.3 ± 1.0 leukocytes/100 EC;) (fig 3)

When adhesion studies were performed using blood from uremic patients, no modifications were observed in adhesion from either basal or 15 min samples. Leukocyte adhesion on EC was increased in samples obtained after 2 hr hemodialysis in both arterial and venous lines (4.2 ± 0.2 and 4.4 ± 0.3 leukocytes/100 EC; $P < 0.005$) respectively. Adhesion values on UEC were similar to those of EC (4.1 ± 0.4 and 4.2 ± 0.3 ; $P < 0.05$) in arterial and venous lines respectively (Fig 3)

These results indicate that uremic EC favours adhesive phenomena in normal leukocytes as can be ob-

served by differences between control blood perfused on normal EC or uremic EC. The impairment observed in adhesive properties of uremic leukocytes at 15 min can be abolished throughout hemodialysis as demonstrate adhesion values after 120 min.

On the other hand, we observed a good correlation between values of leukocyte adhesion under uremic conditions and expression of CD11b in leukocytes from uremic patients with a correlation coefficient of 0.94 and a *r*-squared of 90.1 %. This suggests that CD11b plays an important role in adhesive phenomena in uremic patients.

Discussion

Contact of blood with artificial surfaces may initiate leukocyte inflammatory responses, which are associated with adhesion molecules.^{2,3,12} In previous studies we found modifications in the expression of the leukocyte integrin CD11b during haemodialysis.² We have now extended our studies to analyse modifications in the CD11b phosphorylation levels and the adhesive properties of leukocytes from uremic patients under haemodialysis with a cellulosic membrane. We confirmed the increase in CD11b expression during hemodialysis through this membrane, as observed in previous studies, which correlated with an increased adhesion of leukocytes on endothelial cell monolayers perfused with blood from uremic patients. In addition, our results showed that dialysis with a cellulosic membrane, induces dephosphorylation of the leukocyte integrin CD11b after 120 min on haemodialysis. These results together indicate that both the dephosphorylation and the increase in the expression of CD11b along the hemodialysis procedure occur in response to activation.

In the present study, the expression of CD11b was found to be increased in response to hemodialysis, as we already published.²⁴ We have extensively demonstrated that activation means increased expression of CD11b. In addition, we observed a direct relation between an increased expression of CD11b and dephos-

phorylation of this molecule, more evident in samples obtained from venous line (downstream) than those from arterial line (upstream), indicating that cellulose membrane played an important role in the process of dephosphorylation. We believe that the fact that CD11b dephosphorylates in response to activation is a new and interesting finding. As published by Merrill et al.²⁵, the characteristics of homotypic neutrophil aggregation, mediated by the adhesion molecule CD11b/CD18, differ according to whether activation takes place via intracellular protein kinase C (PKC) inducers or chemoattractants. While the former occurs through phosphorylation of the complex, the latter occurs with no phosphorylation. Therefore, dephosphorylation could be expected in response to activation by the mechanical stress that hemodialysis implies.

Our present results showed that the CD11b integrin is involved in leukocyte adhesion to endothelial cells under flow conditions, as demonstrated by the strong correlation between the expression of CD11b and the degree of leukocyte adhesion on the surface of uremic endothelial. In this study, the introduction of a new condition, consisting of the use of uremic EC as adhesive substrate, approaches our experimental design to a more physiologic setting. In this particular setting we observed increases in the adhesion of leukocytes on the uremic EC monolayers, as previously extensively described by our group.^{22,26-27} These results suggest a priming state in uremic EC similar to the observed in previous studies in leukocytes from uremic patients.

There is evidence that CD11b/CD18 integrin has different ligands²⁸⁻³¹, including iC3b, MMP9, and ICAM-1, among others. ICAM-1 is constitutively expressed, although at low levels, in endothelial cells. Our group has demonstrated that exposure of endothelial cells to sera from uremic patients induces expression of the adhesion molecules VCAM-1, ELAM-1, and ICAM-1.²² Both observations would make ICAM-1 suitable as a ligand for CD11b/CD18 explaining why an increased adhesion is observed on both control and uremic endothelial cells. From our

results, it seems that the expression of CD11b/CD18 would be mandatory on the regulation of the leukocyte adhesion process.

It has been suggested that CD11b phosphorylation on Ser1126 site is required for adhesion events to ICAMs.³² The basal activation would lead to an over-expression of endothelial cells ICAM and ELAM.²² However, when uremic conditions were present in both endothelial cells and leukocytes, the adhesive properties were impaired at baseline whereas CD11b was phosphorylated. Similarly, restoration of values of adhesion throughout was accompanied by CD11b dephosphorylation suggesting that adhesion in uremic patients would not be mediated by ICAM. We could hypothesize another control mechanisms for adhesion in uremic such as changes in the conformation of the extracellular domains of the integrin, influencing its affinity for ligands or a novel selective mechanism allowing to bind CD11b ligands in absence of phosphorylation.³³ However, new studies must be performed in order to elucidate the mechanisms regulating adhesion in uremic conditions.

We conclude that leukocyte activation during haemodialysis through a cellulose membrane occurs with an increase of CD11b expression and a decrease in CD11b phosphorylation levels. The adhesive behaviour of leukocytes on uremic endothelial cell monolayers correlates with CD11b expression but not with the phosphorylation status of the integrin. In addition, we conclude that the priming state observed in leukocytes from uremic patients in previous studies² can be extended to endothelial cells.

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