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Vitamin E-modified filters modulate Jun N-terminal kinase activation in peripheral blood mononuclear cells

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Background. The generation during hemodialysis of activated complement fragments and reactive oxygen species, including nitric oxide (NO), may affect peripheral blood mononuclear cell (PBMC) function. Currently, little is known about signal transduction pathways involved in PBMC activation. Jun N-terminal kinase (JNK) is a novel mitogen-activated protein (MAP) kinase phosphorylated and activated in response to oxidative stress and directly involved in cell activation.

Methods. The present study evaluated the activation of JNK in PBMCs isolated from eight uremic patients undergoing, in a randomized manner, three month-subsequent periods of hemodialysis with a low-flux cellulose acetate (CA) and a vitamin E-modified cellulose membrane (CL-E). After each period of treatment, PBMCs were harvested before (T0), during (T15) and after three hours (T180) of dialysis. At the indicated time points, plasma C5b-9 generation by ELISA and inducible NO synthase (iNOS) gene expression by in situ hybridization were evaluated also. The activation of JNK was studied by Western blotting using a specific monoclonal anti-phospho-JNK antibody, which recognizes the activated form of JNK.

Results. At T0, a significant increase in plasma C5b-9 levels was found in CA patients compared to CL-E-treated patients. During hemodialysis, C5b-9 levels rose more significantly in CA patients than in CL-E patients and returned to baseline values only in CL-E patients. At the same time, in CA patients an increased iNOS gene expression was observed at T180 together with a striking activation of JNK. By contrast, PBMC from CL-E-treated patients showed undetectable levels of phospho-JNK and a significant reduction in iNOS expression. Interestingly, incubation of PBMCs with normal human plasma (10%), activated by contact with a cellulosic membrane, induced a time-dependent increase in JNK phosphorylation that was completely inhibited by blocking complement cascade activation.

Conclusion. Our data suggest that JNK phosphorylation is strikingly increased in PBMCs obtained from CA-treated patients and may represent a key cellular event in PBMC activation during dialysis with bioincompatible membranes. The activation of this signaling enzyme, mediated by active complement fragments and PBMC-dialyzer interaction, can be significantly reduced by the use of vitamin E-coated membrane.

During hemodialysis blood contact with a complement-activating membrane may promote a variety of complex and interrelated events, leading to an acute inflammatory response. In particular, activation of mononuclear cells induces the release of an array of pro-inflammatory compounds into the extracellular environment [1]. It is well known that in the long term these pro-inflammatory molecules may mediate, at least in part, numerous clinical complications observed in hemodialyzed patients [2].

Although the clinical consequences of the immune cell activation have been clarified, the intracellular mechanisms turning on peripheral blood mononuclear cells (PBMC) during dialysis are still largely unknown. Cytosolic tyrosine and serine kinase activation is a key step in the intracellular signaling pathways leading to cell proliferation, migration and cytokine secretion [3]. Several leukocyte functions are mediated by changes in protein tyrosine and serine phosphorylation. Indeed, multiple cellular proteins, including transcription factors, are phosphorylated within seconds after treatment of these cells with different agonists [4]. Most of the interleukins exert their actions on monocytes and lymphocytes through the activation of one or more of the Janus kinases, a family of cytoplasmic tyrosine kinases [5, 6]. On the other hand, the T cell receptor itself is associated with a tyrosine kinase, lck, and activates a variety of serine kinases, including several enzymes of the mitogen-activated protein (MAP) kinase superfamily [7, 8].

The contribution of the complement system in the intracellular events associated with PBMC activation during dialysis remains a debated issue. An increasing body of evidence suggests that terminal complement

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complexes C5b-7, C5b-8 and C5b-9, at sublytic concentrations, can produce non-lethal cell-activating signals [9]. A variety of second messengers, including cyclic adenosine monophosphate (cAMP), inositol phosphate intermediates and arachidonate metabolites, are generated by the terminal complement complexes in several cell types [10, 11]. In addition, C5b-9 has recently been shown to activate different cytoplasmic tyrosine kinases and the ras-raf-MAP kinase signaling cascade [12].

Hemodialysis patients are chronically exposed to oxidative stress, due to an increased production of reactive oxygen species (ROS) and a reduced level of anti-oxidant compounds [13]. ROS have recently been recognized as signaling molecules [14]. Indeed, oxidants can directly activate different cytoplasmic as well as receptor-associated tyrosine kinases [15–17]. In addition, ROS are known to regulate several cell functions through the activation of some stress-activated protein kinases, such as p38 mitogen-activated protein kinase (MAPK p38) and jun-N-terminal kinase (JNK) [18, 19]. In particular, JNK has been shown to mediate cellular stress responses and it is activated by environmental stress factors including ultraviolet light [20], heat shock [21] and pro-inflammatory cytokines [22, 23]. Once activated, JNK in turn can catalyze the phosphorylation of jun, thus promoting its interaction with fos to form an active activator protein-1 (AP-1) [20]. This transcription factor can then modulate the gene expression of several cytokines and growth factors [24].

Recently, a new vitamin E-modified cellulosic membrane (CL-E; Excebrane, Terumo, Japan) has been introduced in dialysis. This modified membrane has been shown to exert a specific and timely scavenging of oxygen free radicals, including nitric oxide (NO), thus reducing the hemodialysis-induced oxidative stress [25]. Moreover, the modified surface seems to improve the biocompatibility profile of this dialyzer [26, 27]. Thus, in the present study we evaluated the modulation of inducible nitric oxide synthase (iNOS) gene expression and JNK activation in PBMC during hemodialysis, and the potential effect of the vitamin E-coated membrane on these two molecular events.

METHODS

Patients

Eight stable hemodialyzed patients (4 men, 4 women, mean age 43.2 years, range 20 to 65 years), having given their informed consent, were enrolled in the study; eight healthy subjects (4 males and 4 females, mean age 41.2 years) matched for sex and age represented the control group. Patients were stabilized on renal replacement therapy for more than six months prior to the study (mean dialytic age 24.2 months; range 12 to 55 months) and were treated with bicarbonate hemodialysis using a cellulose acetate (CA) dialyzer for a mean of 4.0 hours thrice weekly when entering the study. None of them had signs of diabetes, lipid metabolism defects, infection, leukopenia, active immunologic process or malignancy at the time of the study. In addition no medications (anti-inflammatory drugs, corticosteroids, vitamin C) potentially interfering with the parameters under investigation were administered throughout the study. Underlying diseases leading to end-stage renal failure were chronic glomerulonephritis (4 patients), interstitial nephritis (2 patients) and cystic disease (2 patients).

Study design

All hemodialyzed patients, in a randomized manner, were treated for two subsequent periods of three months either with the CA hollow fiber dialyzer (CA 180; membrane surface 1.4 m^2; Althin, Milan, Italy) or with the Excebrane E membrane (CL-E 15; membrane surface 1.5 m^2). After 36 sequential treatments, the patients were switched from CA to CL-E membranes and vice versa for another 36 treatments. Hemodialysis efficiency, as indicated by pre- and post-treatment serum urea and the weight loss over each dialysis session, remained unchanged during the study periods. Dialyzers were not reused. Endotoxin content of the dialysate, as shown by colorimetric Limulus Amebocyte Lysate assay (Coatest Kabi Vitrum, Sweden), was constantly less than 0.05 EU/mL.

White blood cell count

Using an electronic counter, leukocytes and monocyte counts were performed in plasma (ethylenediaminetetraacetic acid; EDTA) samples collected from arterial side of the patient's arteriovenous fistula prior to the onset of dialysis and 15 and 180 minutes thereafter.

Assay for complement fragments C5b-9

The EDTA anticoagulated samples were drawn before dialysis (T0) from the arteriovenous fistula, during dialysis (T15 and T180 min) from the venous lines of the dialyzer and, finally, 4 hours after each dialysis session (T480). The specimens were collected aseptically, centrifuged at 4°C and stored at −70°C until processed. Detection of C5b-9 was based on a double-sandwich enzyme-linked immunosorbent assay (ELISA) system using specific antibodies for the terminal complement complex (Quidel, USA). Mean normal values for C5b-9 were 0.18 ± 0.05 μg/mL (mean ± SD).

Isolation of human PBMC and Western blot analysis

After three months of treatment with CA or CL-E, blood samples (25 mL) were drawn in sterile heparinized vacuum tubes from the patients' arteriovenous fistulae just before (T0) and subsequently from the efferent line of the dialyzer after 180 minutes (T180) of the dialysis session. PBMC were separated on a Ficoll/Hypaque gra-

dient (Pharmacia, Uppsala, Sweden). Cells were washed with 0.9% NaCl solution and remaining erythrocytes destroyed by hypotonic lysis using 0.2% and 1.6% NaCl solutions. Isolated PBMC were lysed with RIPA buffer [1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 5 mmol/L EDTA, 1 mmol/L sodium orthovanadate, 150 mmol/L sodium chloride, 8 μg/mL leupeptin, 1.5% Nonidet P-40, 20 mmol/L Tris-HCl, pH 7.4] and centrifuged at 10,000 × *g* for 30 minutes at 4°C. The lysate supernatant was collected, and its protein content determined using the Bio-Rad reagent (Bio-Rad, Munich, Germany). Twenty micrograms of protein from each lysate were mixed with sodium dodecyl sulfate (SDS) sample buffer, aliquots were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE; 7.5% acrylamide) and electrophoretically transferred onto a nitrocellulose filter. The filter was blocked overnight at room temperature (RT) with 2% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) containing 0.1% Tween-20 (TBS) and incubated with monoclonal anti-phosphoJNK or anti-phospho p38 MAP kinase antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at RT for four hours. The membranes were washed twice in TBS and incubated for two hours at RT with horseradish-peroxidase-conjugated sheep anti-mouse IgG at 1:1500 dilution in TBS. The membranes were washed three times at RT in TBS and then once with 0.1% SDS in PBS. The ECL enhanced chemiluminescence system (Amersham, Little Chalfont, UK) was used for detection. The same membranes were then stripped and immunoblotted again with anti-human JNK polyclonal antibody (Santa Cruz Biotechnology) at RT for four hours. The immune complexes were detected on the membranes as described above.

In situ hybridization

Inducible nitric oxide synthase (iNOS) gene expression in cultured PBMC was studied by in situ hybridization. For this purpose, cytospins of freshly isolated PBMC were prepared onto polylysine-coated slides, dried briefly on a hot plate (80°C) and fixed for 20 minutes in 4% paraformaldehyde.

A 1000 bp fragment from human iNOS cDNA (a kind gift of Dr. Pinzani, University of Florence) was subcloned into pGEM3Zf. After linearization of the plasmid with the appropriate restriction endonuclease, T7 or SP6 RNA-polymerases (Boehringer Mannheim, Mannheim, Germany), respectively, were employed to obtain run off transcripts of either the antisense (complementary to mRNA) or sense (negative control) strands. Transcription and labeling of RNA probes was performed as described [28]. Briefly, 80 μCi of [^{35}S]uridine-5′-(α-thio)-triphosphate (specific activity 1250 Ci/mmol; Amersham) were added to a 10 mL reaction mixture (0.5 mmol/L each of adenosine-, cytosine- and guanosine-5′-triphosphate/1 mmol/L dithiothreitol (DTT)/10 units human placental RNase inhibitor/6 mmol/L $MgCl_2$/10 mmol/L Tris-HCl, pH 7.5/2 mmol/L spermidine/10 mmol/L NaCl) including 1 μg of linearized plasmid and 16 units of either SP6 or T7 RNA polymerase. The reaction was allowed to proceed for 60 minutes at 38°C. The plasmid DNA was removed by digestion with 25 mg/mL RNase-free DNase I in a mixture containing 2.5 mg/mL of yeast tRNA and 10 units of RNase inhibitor for 10 minutes at 37°C. Free ribonucleotides were removed by phenol-chloroform extraction followed by ethanol precipitation. RNA probe were then diluted in hybridization buffer, stored at −80°C and used within four weeks. The specific activity usually obtained was 1.2 to 1.4 × 10^9 cpm/μg of ^{35}S-labeled RNA probe.

Prehybridization, hybridization, removal of non-specifically bound probe by RNase A digestion, and further washing procedures were performed for both sense and antisense probes as described previously [28]. Autoradiography was performed by dipping the dehydrated slides into Ilford G5 nuclear emulsion (Ilford, Mobberley Cheshire, UK). The exposed slides were developed using Kodak D19 developer (Kodak, Hemel Hampstead, UK), counter stained in eosin-hematoxylin and, finally, mounted.

Inducible nitric oxide synthase gene expression (silver grains) was quantified by a computer-based morphometric analysis system (Optilab Pro 2.6.1 Software; Graftek, Villanterio, PV, Italy), using a densitometric procedure.

In vitro study

Mini-dialyzer modules containing CA, polyarylethersulfone (Arylane, Hospal, France), polyacrylonitrile (AN 69, Hospal, France) or CL-E membrane (fiber length 18 cm, number of fibers 340, effective membrane area 300 cm^2) were used for in vitro dialysis. Mini-modules and tubings were primed with normal saline before use. Twenty milliliters of heparinized whole blood, withdrawn from healthy subjects, were uniformly passed through each module at a Qb = 1 mL/min for 15 minutes in the presence or in the absence of EDTA at the indicated concentrations. Plasma samples were collected after dialysis and used to stimulate freshly isolated PBMC. For this purpose the cells were pre-incubated in serum-free RPMI overnight and then exposed to the plasma (10% vol/vol) obtained after the in vitro dialysis. At the indicated time points the PBMC were lysed in RIPA buffer as previously described and used for Western blot studies. In a separate set of experiments, freshly isolated PBMC were resuspended in serum free RPMI at 10^6 cells/mL, passed through the modules and lysed in RIPA buffer. Experiments were performed with blood from three healthy subjects.

Statistics

All results have been given as mean ± standard deviation (SD). Significance was assessed using the paired or

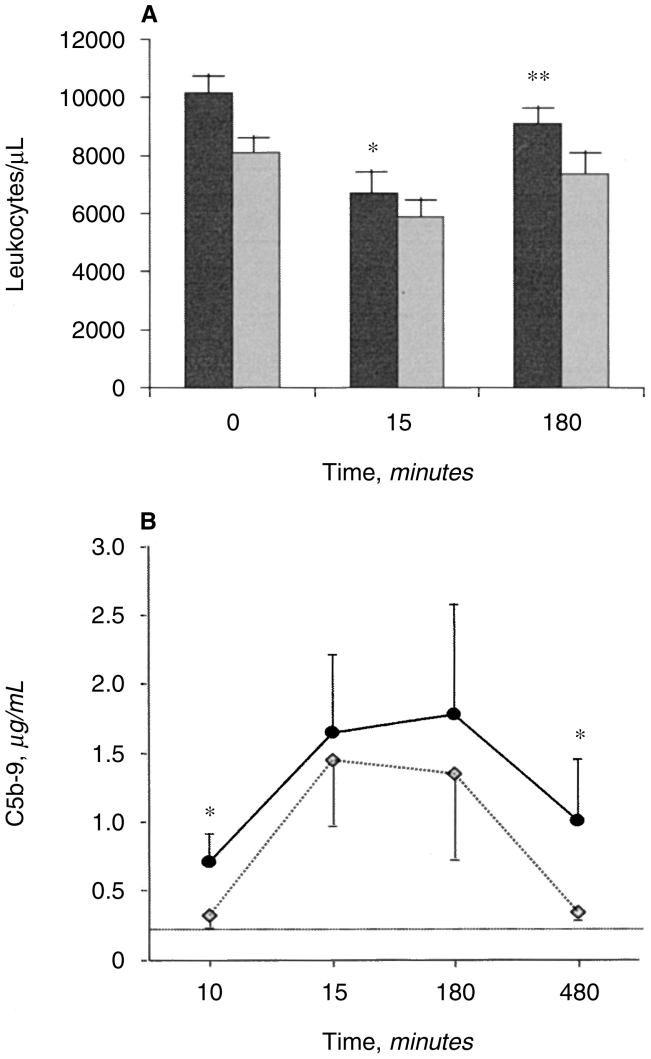

Fig. 1. (*A*) Leukocyte counts during hemodialysis with a cellulosic membrane (CA; ■) and Excebrane-E (CL-E; ▩). *$P < 0.05$ vs. CA T0; **$P < 0.05$ vs. CA T15. (*B*) C5b-9 generation during hemodialysis with CA (●) and CL-E (○). Plasma concentrations of C5b-9 were measured by ELISA as described in the **Methods** section. The dotted line represents the mean C5b-9 concentration in the control group (0.18 ± 0.05 μg/mL); *$P < 0.005$ vs. CL-E membrane.

unpaired Student *t* test, as appropriate. Differences were considered to be significant when $P < 0.05$.

RESULTS

Leukocyte counts and C5b-9 generation during dialysis

Circulating leukocyte counts dropped after 15 minutes of hemodialysis both in CA- and CL-E–treated patients, although the reduction was statistically significant ($P < 0.005$) only in the CA group (Fig. 1A). The number of monocytes showed a similar pattern for both CA (T0 654 ± 122, T15 331 ± 167, T180 631 ± 238 monocytes/μL) and CL-E membrane (T0 686 ± 44, T15 401 ± 203, T180 540 ± 183 monocytes/μL).

Long-term hemodialysis patients treated with CA membranes showed increased predialysis (T0) plasma C5b-9 levels as compared to CL-E treated patients and normal controls ($P < 0.005$; Fig. 1). Moreover, a rapid significant increase of C5b-9 levels occurred at T15 in both groups as compared to baseline values. Thereafter, the generation of the terminal complement complex reached a plateau, which lasted up to 180 minutes after the beginning of dialysis (Fig. 1). Interestingly, at T480, plasma C5b-9 concentration rapidly returned to baseline values in CL-E patients, whereas a slower fall-off was observed in CA-treated patients. Indeed, at T480, C5b-9 in this group of patients remained significantly ($P < 0.005$) higher as compared to patients in hemodialysis with CL-E filters and controls (Fig. 1B).

Effect of CL-E membrane on PBMC activation

To evaluate the level of activation of circulating mononuclear cells, we investigated PBMC iNOS expression during dialysis with CA and CL-E membranes [29, 30]. An extremely high iNOS mRNA expression was observed, by in situ hybridization, in PBMC isolated from CA treated patients, in particular after three hours of dialysis (230,875 ± 32,152 AU/pixel; Fig. 2A). Interestingly, iNOS expression was dramatically reduced in PBMC from patients undergoing dialysis with the vitamin E-coated membrane (50,689 ± 10,253 AU/pixel, $P < 0.01$ vs. CA treatment; Fig. 2B).

Dialysis-dependent stress kinases modulation

Oxidative stress and cytokines generated during HD may activate JNK [18, 19]. Interestingly, after 180 minutes of dialysis patients treated with CA membranes presented a striking increase in the phosphorylation of both JNK-1 and JNK-2, the two main isoforms of the enzyme (Fig. 3). On the other hand, undetectable levels of phospho-JNK were observed when the patients were switched to CL-E (Fig. 3). Either CA or CL-E treatment did not influence the JNK protein expression (Fig. 3). The dialysis-induced changes in protein phosphorylation were specific for JNK and were not observed for the other stress-activated kinase, MAPK p38 (Data not shown).

PBMC activation in vitro

To investigate the mechanisms underlying JNK activation during hemodialysis we moved to an in vitro dialysis system. First, the in vivo observation of a reduced JNK phosphorylation by CL-E was confirmed. Indeed, normal plasma that had been previously dialyzed in vitro with CA membrane induced a striking phosphorylation only of JNK-1 in freshly isolated PBMC, whereas CL-E dia-

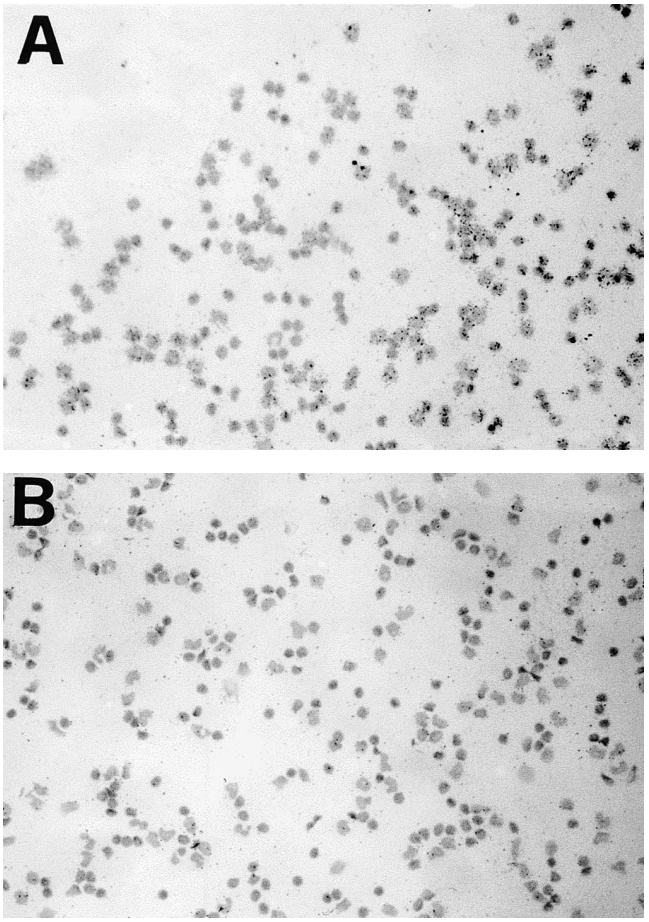

Fig. 2. Inducible nitric oxide synthase (iNOS) gene expression in peripheral blood mononuclear cells (PBMC) of hemodialysis patients treated with different membranes. iNOS gene expression was studied by in situ hybridization as described in the **Methods** section. Bright field photomicrographs of PBMC isolated after 180 minutes of dialysis from patients treated with CA (*A*) or CL-E (*B*) membranes. CA treatment induces a clear up-regulation of iNOS mRNA, whereas the treatment with CL-E reduces it. Photomicrographs are representative of eight patients.

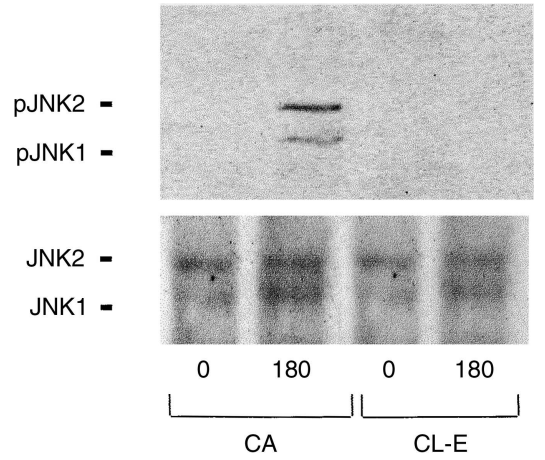

Fig. 3. Modulation of JNK phosphorylation induced by dialysis with CA or CL-E membrane. PBMC were isolated and lysed at T0 and T180. Phosphorylated JNK (top) was studied by western blotting using a specific monoclonal anti-phosphoJNK antibody as described in the **Methods** section. The same membranes were then stripped and immunoblotted again with anti-JNK monoclonal antibody (bottom). Treatment with CA, but not with CL-E, induces a striking increase of both JNK1 and JNK2 phosphorylation at T180. Data are representative of eight patients.

lyzed plasma did not change the JNK phosphorylation level, as compared to untreated PBMC (Fig. 4).

To evaluate the influence of the complement system activation in this signaling event, C5b-9 generation was blocked by adding EDTA (10 mmol/L) during the in vitro dialysis with CA membrane. EDTA significantly reduced C5b-9 levels in the dialyzed plasma (without EDTA 3.3 ± 0.24 μg/mL; with EDTA 0.3 ± 0.01 μg/mL; $P < 0.001$) and caused a marked reduction in the ability of CA-dialyzed plasma to induce JNK phosphorylation (Fig. 5). In addition, plasma dialyzed in vitro with two synthetic membranes, well-known to cause a low C5b-9 generation, was unable to induce any significant change in JNK phosphorylation (Fig. 6).

Finally, to evaluate the role of cell-membrane interaction in HD-induced JNK activation, PBMC resuspended in serum free RPMI were passed through the dialysis modules. The direct contact of PBMC with CA membrane caused a striking phosphorylation of both JNK-1 and JNK-2 that was not present in the cells incubated with CL-E (Fig. 7).

DISCUSSION

There is an increasing body of evidence suggesting that lymphomononuclear cell activation and subsequent cytokine expression may play a key role in the pathogenesis of several dialysis-related pathological conditions, including malnutrition, anemia, accelerated atherosclerosis and immune dysfunction [31]. While the importance of dialyzer biocompatibility in the prevention of several dialysis complications is now clearer, the cellular and molecular mechanisms underlying membrane-induced immune cell activation are still largely unclear.

The present study demonstrates, to our knowledge for the first time, that dialysis with cellulosic membrane can induce the phosphorylation and the subsequent activation of JNK. This intracellular event is temporarily related to a striking activation of circulating mononuclear cells, as suggested by the increased iNOS gene expression, and with a significant increase in C5b-9 generation. Interestingly, in vitro inhibition of complement cascade priming markedly reduces the JNK phosphorylation.

Complement activation is a major feature in bioincompatibility phenomena. The presence of high C5b-9 plasma levels just prior to dialysis suggests that a chronic complement activation state exists in CA-treated pa-

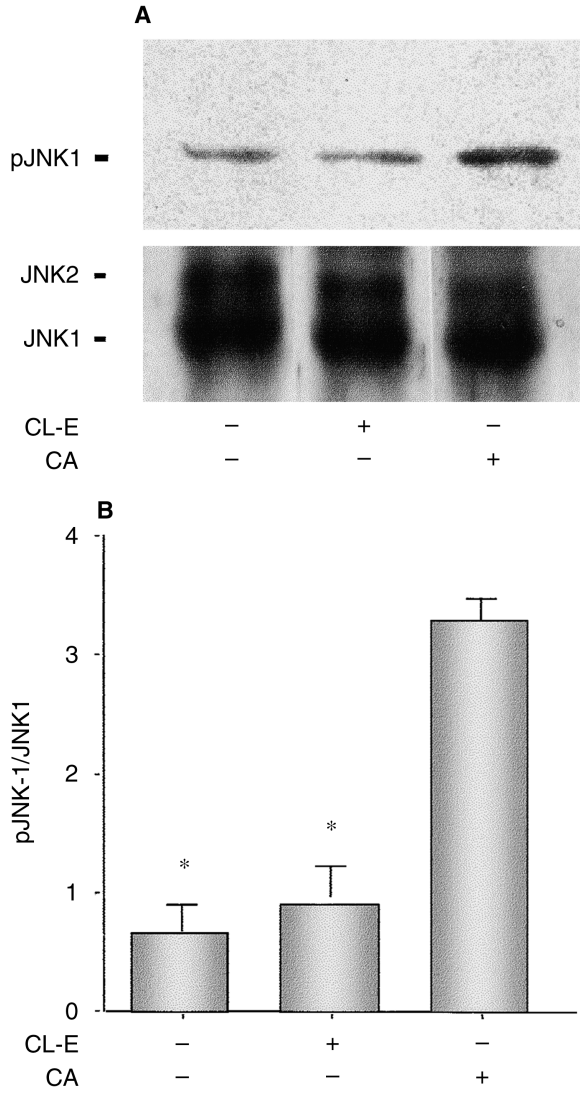

Fig. 4. (*A*) In vitro effect of CA and CL-E membranes on JNK phosphorylation. Twenty milliliters of heparinized whole blood, drawn from healthy subjects, were dialyzed in vitro with CA or CL-E, as described in the **Methods** section. Plasma samples were collected after dialysis and used to stimulate freshly isolated PBMC. For this purpose the cells were pre-incubated in serum-free RPMI overnight and then exposed for 15 minutes to the plasma (10% vol/vol) obtained after the in vitro dialysis. JNK phosphorylation (A) was evaluated. The same membranes were then stripped and immunoblotted again with anti-JNK monoclonal antibody (lower panel in A). Data are representative of three independent experiments. (*B*) Densitometry analysis was performed on the blots by a computer-assisted image analysis system. The levels of p-JNK were normalized to the total content of the enzyme and expressed as a ratio (p-JNK/JNK). $*P < 0.001$ vs. CA.

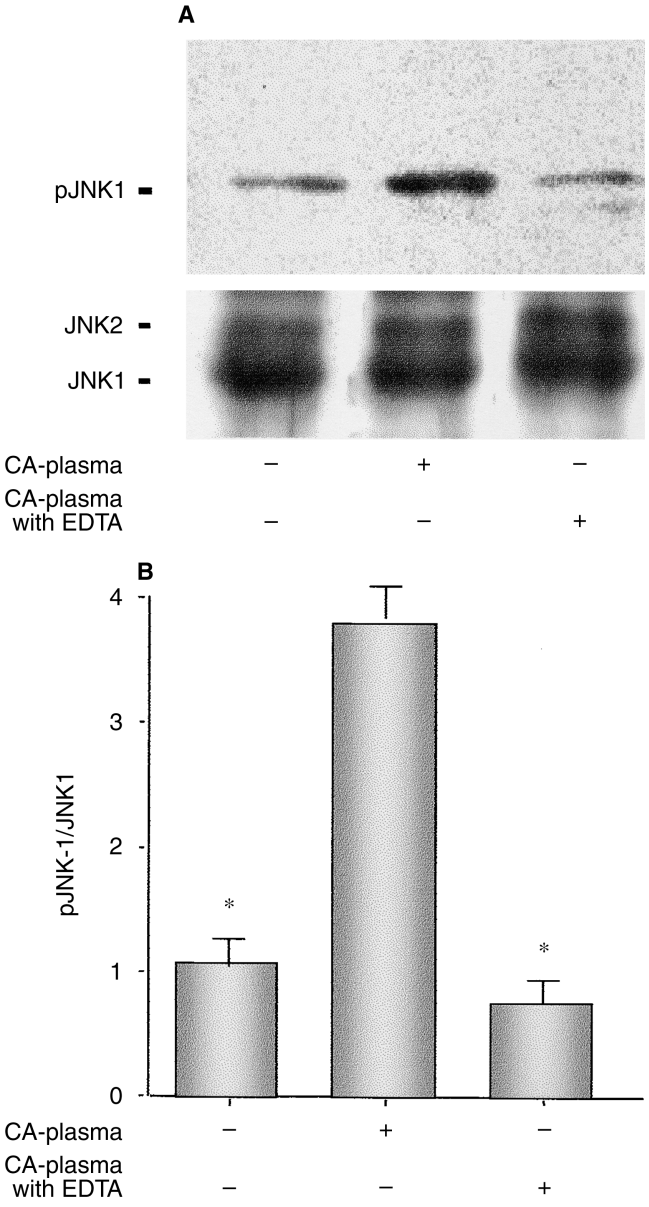

Fig. 5. (*A*) Complement-dependent JNK phosphorylation during in vitro dialysis. Twenty mL of heparinized whole blood, drawn from healthy subjects, was dialyzed in vitro in the presence or in the absence of EDTA (10 mmol/L). Plasma samples were collected after dialysis and used to stimulate freshly isolated PBMC. For this purpose the cells were pre-incubated in serum-free RPMI overnight and then exposed for 15 minutes to the plasma (10% vol/vol) obtained after the in vitro dialysis. JNK phosphorylation (A) was evaluated as described in the **Methods** section. The same membranes were then stripped and immunoblotted again with anti-JNK monoclonal antibody (A, lower panel). Data are representative of three independent experiments. (*B*) Densitometric analysis was performed on the blots by a computer-assisted image analysis system. The levels of p-JNK were normalized to the total content of the enzyme and expressed as a ratio (p-JNK/JNK). $*P < 0.001$ vs. CA.

tients in the interdialytic period. This might be explained by the relative inefficacy of these membranes to eliminate middle molecules such as factor D, a serine esterase with a molecular weight of 23 kD able to trigger the activation of the alternative pathway [32]. Moreover, a marked lowering of serum S protein (Vitronectin), a control protein of C5b-9 complex, has been reported in hemodialyzed patients [33]. Thus, the elevated concentration of factor D might be responsible for the enhanced activation of the alternative pathway in uremic patients and account for a continuous generation of C5b-9, not sufficiently controlled by S protein [34]. Activated com-

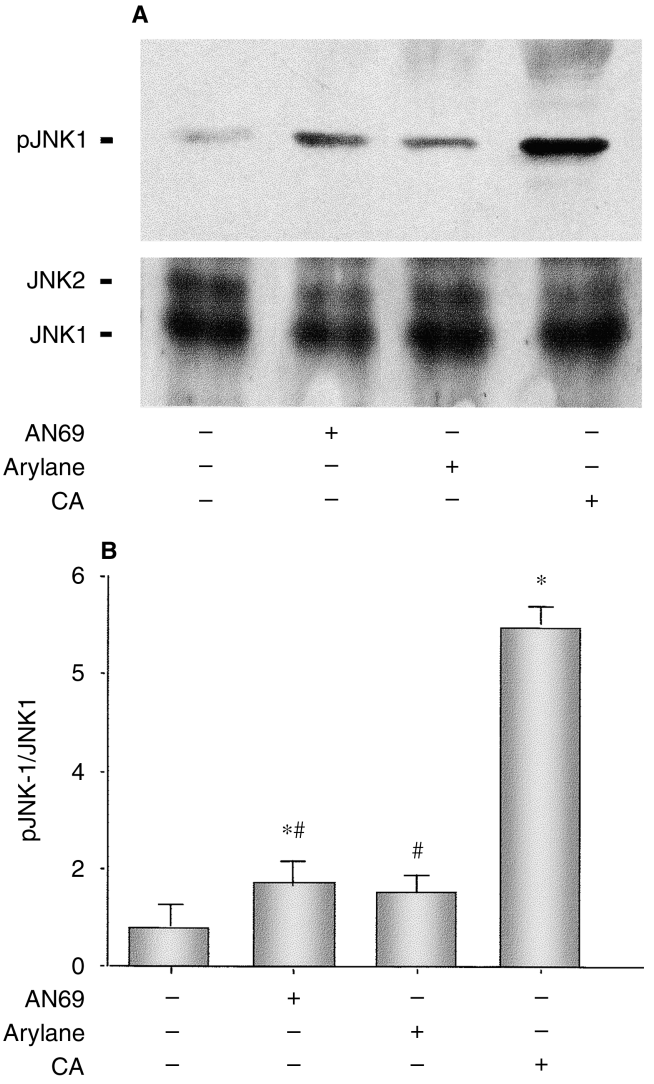

Fig. 6. (*A*) In vitro effect of different dialytic membranes on JNK phosphorylation. Twenty milliliters of heparinized whole blood, drawn from healthy subjects, were dialyzed in vitro with CA, AN 69 and Arylane, as described in the **Methods** section. Plasma samples were collected after dialysis and used to stimulate freshly isolated PBMC. To this purpose the cells were pre-incubated in serum-free RPMI overnight and then exposed for 15 minutes to the plasma (10% vol/vol) obtained after the in vitro dialysis. JNK phosphorylation (A) was evaluated as described. The same membranes were then stripped and immunoblotted again with anti-JNK monoclonal antibody (A, lower panel). Data are representative of three independent experiments. (*B*) Densitometric analysis was performed on the blots by a computer-assisted image analysis system. The levels of p-JNK were normalized to the total content of the enzyme and expressed as a ratio (p-JNK/JNK). $*P < 0.001$ vs. basal; $\#P < 0.001$ vs. CA.

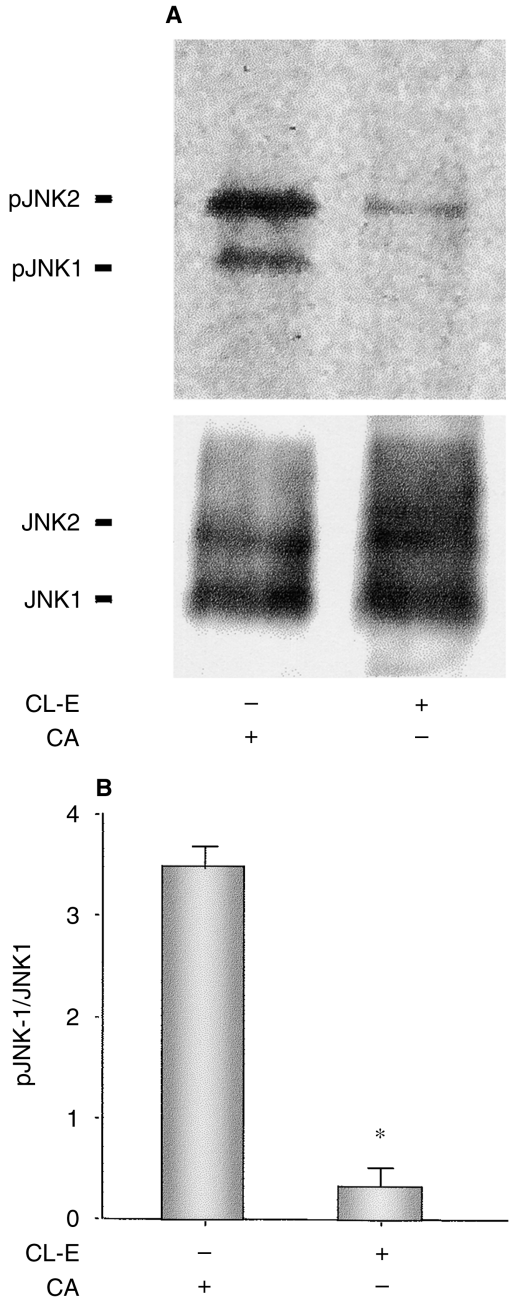

Fig. 7. (*A*) Direct membrane effect on JNK phosphorylation. Freshly isolated PBMC obtained from healthy subjects were resuspended in serum-free RPMI at 10^6/mL dialyzed in vitro as described in the **Methods** section, and lysed in RIPA buffer. JNK phosphorylation (A) was evaluated. The same membranes were then stripped and immunoblotted again with anti-JNK monoclonal antibody (A, lower panel). Data are representative of three independent experiments. (*B*) Densitometric analysis was performed on the blots by a computer-assisted image analysis system. The levels of p-JNK were normalized to the total content of the enzyme and expressed as a ratio (p-JNK/JNK). $*P <$ 0.001 vs. CA.

plement components and, in particular the terminal complement complex, can modulate several cell functions through the activation of different signaling pathways [9]. Sublytic doses of C5b-9 have been shown to induce inositol phosphate generation, calcium influx and cytoplasmic protein tyrosine phosphorylation [9–12]. Interestingly, Viedt et al recently reported that C5b-9 causes an increased production of reactive oxygen species and a significant up-regulation of interleukin-6 (IL-6) gene expression [35]. This pro-inflammatory cellular effect of the complement terminal complex is completely inhib-

ited by the antioxidant N-acetylcysteine, suggesting that oxidative stress may represent one of the main mediators of C5b-9 signaling and subsequent modulation of cellular functions [35].

Oxidative stress during dialysis is particularly deleterious, since dialysis patients present a significant reduction of the anti-oxidant defense, mainly due to the loss of vitamins C and E [13]. Circulating immune cells are extremely sensitive to this stimulus. Indeed, ROS can activate several intracellular signaling pathways in lymphomononuclear cells. In this scenario, the stress-activated kinases represent a primary target [14]. This recently recognized family of MAP kinases, including JNK and p38 MAPK, seems to play a key role in the signaling system leading to cell activation in response to a broad range of external stimuli [18–22]. The signaling through JNK causes the activation of different transcription factors, including AP1 and ATF2, which in turn can induce an increased transcription of cytokines and growth factors, a hallmark of bioincompatibility [24]. JNK present two main isoforms, JNK-1 and JNK-2 transcribed by two independent genes, with mostly overlapping functions in immune cells [36–38]. In our in vivo study we observed the phosphorylation of both isoforms. Interestingly, the in vitro stimulation of PBMC with plasma dialyzed using CA membranes caused the phosphorylation only of JNK-1. On the other hand, the direct contact of PBMC with the cellulosic membrane in a complement-free setting induced the phosphorylation of both isoforms and in particular of JNK-2. This observation suggests a different pathway of activation for the two JNK isoforms during hemodialysis.

Since oxidative stress might be significantly involved in the pathogenesis of the observed pro-inflammatory cellular events, we investigated the ability of a newly introduced vitamin E-coated cellulosic membrane to modulate this phenomenon both in vivo and in vitro. Galli et al recently demonstrated that this modified membrane reduces NO plasma level [25]. Moreover, Girndt et al reported that, despite being based on a cellulosic backbone, CL-E presents a biocompatibility profile similar to one of the less complement-activating synthetic membranes, polyamide [27]. Indeed, vitamin E-coated membrane as well as polyamide improved lymphomononuclear cell function [27]. In addition CL-E, but not polyamide, reduced acute production of IL-6 during dialysis [27]. Interestingly, in the present study the use of CL-E significantly reduced the C5b-9 generation, almost completely blocked iNOS expression, and abolished JNK activation in vivo and in vitro. Noteworthy, the observed reduction in C5b-9 generation is not proportional to the degree of iNOS and JNK inhibition, suggesting that this is not the main point of action of vitamin E. However, the interaction of the terminal complement complex as well as the cellulosic membrane itself with circulating immune cells may lead to a significant release of ROS [35]. It is conceivable, then, that the high local concentration of vitamin E may dramatically reduce the oxidative load, as reported by Tarng et al [26]. In turn, this can reduce JNK activation and eventually iNOS gene expression. Indeed, in the 5′ flanking sequence of the iNOS gene there are two AP-1 binding sites whose relevance in iNOS gene modulation in response to different cytokines has been recently reported [39, 40]. However, on the basis of our in vitro and in vivo data we cannot exclude the possibility that complement activation or membrane-cell interaction might induce iNOS gene expression in a JNK-independent manner.

In conclusion, our data suggest that JNK phosphorylation is strikingly increased in PBMC obtained from CA-treated patients and may represent a key cellular event in PBMC activation during dialysis with bioincompatible membranes. The activation of this signaling enzyme, mediated by active complement fragments and PBMC-dialyzer interaction, can be significantly reduced by the use of vitamin E-coated membrane. Therefore, the development of this "third generation" dialyzer might provide a specific and efficient tool to prevent the continuous immune activation induced by dialysis and its long-term clinical consequences in hemodialyzed patients.

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