Coagulation Cascade Activation Causes CC Chemokine Receptor-2 Gene Expression and Mononuclear Cell Activation in Hemodialysis Patients

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Priming of the coagulation cascade during hemodialysis (HD) leads to the release of activated factor X (FXa). The binding of FXa to its specific receptors, effector protease receptor-1 (EPR-1) and protease-activated receptor-2 (PAR-2), may induce the activation of peripheral blood mononuclear cells (PBMC) and promote a chronic inflammatory state that is responsible for several HD-related morbidities. In the attempt to elucidate the mechanisms underlying the coagulation-associated inflammation in HD, 10 HD patients were randomized to be treated subsequently with a cellulose acetate membrane (CA) and Ethylen-vinyl-alcohol (EVAL), a synthetic membrane that has been shown to reduce FXa generation. At the end of each experimental period, surface FXa and thrombin receptors (EPR-1 and PAR-1-2, -4) and CCR2 (monocyte chemoattractant protein-1 receptor) gene expression in isolated PBMC were examined. The ability of dialytic membranes to activate protein-tyrosine kinases and the stress-activated kinase JNK and to modulate the generation of terminal complement complex (TCC) was also investigated. EPR-1 and PAR-2 and -4 mRNA expression, barely detectable in normal PBMC, were significantly upregulated in HD patients, particularly in those who were treated with CA. A striking increase of tyrosine-phosphorylated proteins and JNK activation was observed at the end of HD only in CA-treated patients. Simultaneously, an increased gene expression for both splicing isoforms of CCR2, A and B, only in PBMC from CA-treated patients was demonstrated. The increased CCR2 mRNA abundance was followed by a significant increase in its protein synthesis. The high expression of CCR2 was associated with an increased generation of plasma TCC and a significant drop in leukocyte and monocyte count. By contrast, EVAL treatment slightly lowered TCC generation and normalized leukocyte and monocyte count.

In vitro FXa induced CCR2 A and B expression and JNK activation in freshly isolated PBMC. FXa-induced CCR2 mRNA expression was completely abolished by JNK and tyrosine kinase inhibition. In conclusion, these data suggest that subclinical clotting activation may cause an increased CCR2 gene and protein expression on uremic PBMC, contributing to HD-related chronic microinflammation. The use of the less coagulation-activating membrane, EVAL, may reduce PBMC activation through the modulation of the stress-activated kinase JNK.

Blood interaction with cellulosic membranes during hemodialysis (HD) may cause the activation of several biologic systems, including complement and coagulation cascades (1). Despite clinically effective anticoagulation, priming of the clotting system commonly occurs during HD, leading to the release of several proteases, including thrombin and activated factor X (FXa) (2). FXa has been shown to induce cell proliferation (3), PDGF-A and B chain gene expression (4), and IL-6 release in several cell lines (5). Altieri et al. (6) identified effector protease receptor-1 (EPR-1) as the main FXa receptor on leukocytes and endothelial cells. However, recent observations support the hypothesis that FXa may induce cell activation primarily interacting with protease-activated receptor-2 (PAR-2). PAR-2 belongs to a growing family of G protein–coupled receptors activated by limited proteolysis (7–9). Although Vu et al. (10) characterized the first of these receptors in the early 1990s, very little is known about the effect of HD on PAR expression in peripheral blood mononuclear cells (PBMC).

Leukocyte activation during HD is the central event in the pathogenesis of bioincompatibility, but the mechanisms of this phenomenon remain unclear. Monocyte chemoattractant protein-1 (MCP-1), a member of the CC family of chemokines, has been suggested to play a pivotal role in HD-induced mononuclear cell activation (11,12). We and others (13,14) reported that thrombin may induce MCP-1 expression in different cell lines, suggesting a close relationship between coagulation and inflammatory response. MCP-1 effects are mediated by a specific cell-surface receptor, CC chemokine receptor 2 (CCR2), mainly expressed in monocytes, basophils, and certain subsets of T cells (15,16). There are several pieces of experimental evidence...
that CCR-2 may play a key role in the pathogenesis of atherosclerosis (17,18). Although the potential inflammatory effects of clotting proteases now are clear, the question of whether a reduced priming of coagulation cascade may improve HD-induced PBMC activation is still unanswered. To address this issue, we evaluated in vivo CCR2 gene expression and PBMC activation in uremic patients who were treated with low-flux cellulose acetate (CA) or Ethylen-vinyl-alcohol (EVAL) membrane, a synthetic membrane characterized by a reduced thrombogenic activity (19).

Materials and Methods

Patients

Ten stable HD patients (five men and five women; mean age 45.3; range 25 to 67 yr), having given their informed consent, were enrolled in the study; 10 healthy subjects (five men and five women; mean age 43.2; range 25 to 62 yr) who were matched for gender and age represented the control group. Patients were stabilized on renal replacement therapy for >6 mo before the study (mean dialytic age 24.2; range 12 to 55 mo) and were treated by bicarbonate HD using a CA dialyzer for a mean of 4.0 h thrice weekly when entering the study. None of them had signs of infection, active immunologic processes, or malignancy at the time of the study. Underlying diseases leading to end-stage renal failure were chronic glomerulonephritis (five patients), interstitial nephritis (three patients), and cystic disease (two patients).

Study Design

All HD patients, in a randomized manner, were treated for two subsequent periods of 3 mo either with a CA hollow-fiber dialyzer (CA 180; Althin, Milan, Italy; membrane surface 1.4 m²) or with EVAL membrane (KF101–1.6; Kuraray, Tokyo, Japan; membrane surface 1.6 m²). After 36 sequential treatments, the patients were switched from CA to EVAL and vice versa for another 36 treatments. HD efficiency, as indicated by urea reduction rate, remained unchanged during the study periods. Dialyzers were not reused. Endotoxin content of the dialysate, as shown by colorimetric Limulus Amebocyte Lysate assay (Coastal Kabi Vitrum, Stockholm, Sweden), was constantly <0.05 EU/ml.

Isolation of Human PBMC and Western Blot Analysis

At the end of each treatment period with CA or EVAL, blood samples (25 ml) were drawn in sterile, heparinized vacuum tubes from the patients’ arteriovenous fistulae just before the second HD session of the week (T0) and subsequently from the efferent line of the dialyzer after 15 (T15) and 180 min (T180) of the same dialysis session. PBMC were separated on a Ficoll/Hypaque gradient (Pharmacia, Uppsala, Sweden). Isolated PBMC were lysed with RIPA buffer (1 mM NaCl solutions. Isolated PBMC were lysed with RIPA buffer (1 mM NaCl, 1 mM MgCl₂, 1 mM dNTP, 20 μM of RNase inhibitor, 2.5 mM oligo (dI), and 100 μM of Unolone murine leukemia virus reverse transcriptase was incubated at 42°C for 30 min and then heated to 95°C for 5 min to inactivate the enzyme activity and to denature RNA-CDNA hybrids. PCR was performed with separate sets of oligonucleotide primers, specific for each gene studied. For CCR2, we used two sets of primers that recognize the two splicing isoforms of the receptor, CCR2A and CCR2B (16):EPR-1 5′-TTAAGCGCTGACTGACGCTG-3′, 5′-TGTTAAACAGCTACGCTGCCTC-3′; PAR-1 5′-CTCAGACGCTGTAGAATCGAC-3′, 5′-GGTACACTTCATGCATACTGCC-3′; PAR-2 5′-TGTTTTGCAAGTTAAAGCCC-3′, 5′-GGGAGATGCCAAGTTACG-3′; PAR-3 5′-CTCAGACGAGGCGGCAGGAC-3′, 5′-CACGCCAGGGCCACGGAGGTCC-3′; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) 5′-TGTTATCCTGGAAGACCTGATAC-3′, 5′-ATGCCATGAGCTCCGTCAGC-3′; PAR-2 5′-TGTTTTGCAAGTTAAAGCCC-3′; PAR-3 5′-CTCAGACGAGGCGGCAGGAC-3′, 5′-CACGCCAGGGCCACGGAGGTCC-3′; PAR-4 5′-CTCAGACGAGGCGGCAGGAC-3′, 5′-CACGCCAGGGCCACGGAGGTCC-3′; PAR-5 5′-CTCAGACGAGGCGGCAGGAC-3′, 5′-CACGCCAGGGCCACGGAGGTCC-3′.

EPR-1, PAR-1, PAR-2, PAR-4, and GAPDH cDNA amplification was performed in two separate sets of reactions at a final concentration of 1× PCR buffer, 1.5 mM MgCl₂, 200 μM dNTP, 0.15 μM PAR-2 primers or 0.15 μM GAPDH primers, and 1.25 μU of AmpliTaq DNA polymerase (Perkin Elmer, Monza, Italy) in a total volume of 50 μl. The amplification profile involved denaturation at 95°C for 1 min, primer annealing at 61°C for PAR-2 or at 55°C for GAPDH, and extension at 72°C for 1 min. PCR products were electrophoresed in 1.6% agarose gel in Tris borate/EDTA buffer, loading 10 μl of either PAR-2 or GAPDH PCR products for each sample. Intensity of the PAR-2 and GAPDH bands was quantified by computer-assisted densitometry (Optilab 2.6.1; Graftek, Villanterio, Italy). Results were expressed as PAR-2 to GAPDH ratios.

Assay for Terminal Complement Complex

EDTA anticoagulated samples were drawn before dialysis (T0) and after 15 min (T15), 180 min (T180), and 480 min (T480) of HD. The specimens were collected aseptically, centrifuged at 4°C, and stored at −70°C until processed. Detection of terminal complement complex (TCC) was based on a double-sandwich ELISA system using a specific antibody for the activated complement component (Quidel, San Diego, CA). Mean normal value was 0.16 ± 0.07 μg/ml.

In Vitro Study

Twenty milliliters of heparinized whole blood, drawn from healthy subjects, were used to isolate PBMC as described above. The cells were resuspended at 10⁶/ml, preincubated in serum-free RPMI overnight, and then exposed to PMA (10 nM; Calbiochem, La Jolla, CA). At the
indicated time points, the PBMC were lysed in RIPA buffer as described previously and used for Western blot studies. In a separate set of experiments, freshly isolated PBMC were resuspended in serum-free RPMI at 10⁶ cells/ml, stimulated with FXa for the indicated time period, and lysed in guanidium isothiocyanate. Total RNA then was extracted by the single-step method and used for RT-PCR as described above. Experiments were performed with blood from three different healthy subjects.

**Statistical Analyses**

All results are given as mean ± SD. Significance was assessed using paired or unpaired t test as well as repeated measures ANOVA, as appropriate. Differences were considered to be significant at \( P < 0.05 \).

**Results**

**Effect of Dialytic Membranes on PBMC Activation and CCR2 Gene Expression**

The first step of the study was to investigate the ability of the two membranes to activate circulating mononuclear cells. To this purpose, we evaluated whether dialysis treatment with either CA or EVAL may induce the activation of one or more cytoplasmic tyrosine kinases. In addition, we investigated the phosphorylation and subsequent activation of JNK, a stress-activated kinase that plays a key role in several leukocyte functions. *In vivo* interaction of PBMC with CA membrane induced an increase in cytoplasmic levels of tyrosine-phosphorylated proteins, an indirect measure of tyrosine kinase activation, that peaked at the end of the dialysis session (Figure 1, A and B). During the EVAL treatment, however, the low levels of tyrosine phosphorylated proteins observed at T0 remained unchanged throughout the dialysis session, with only a slight increase at the end of HD (Figure 1, A and B). Simultaneously, at T180, we observed a significant increased phosphorylation of both JNK isoforms in CA-treated patients but not in those who underwent dialysis with EVAL (Figure 1, C and D).

To investigate whether the activation of intracellular signaling pathways influenced the phenotype of circulating mononuclear cells, we evaluated by RT-PCR the expression of CCR2, the specific MCP-1 receptor. Because there are two splicing isoforms of CCR2, A and B, which have been hypothesized to

![Figure 1](image-url)
play a different role in cell activation, we used two sets of primers that are able to recognize CCR2 A and B (16). A weak expression of both isoforms was observed in normal PBMC (Figure 2A). By contrast, PBMC from CA-treated uremic patients presented an increased mRNA expression for the CCR2A at the beginning of and during the dialysis session (Figure 2, B and C). Conversely, CCR2A gene expression was barely detectable in PBMC from EVAL-treated patients (Figure 2, B and C). Similarly, at all times of dialysis, the CCR2B gene expression was markedly upregulated in patients who were treated with CA. In contrast, PBMC from patients who underwent dialysis with EVAL membrane showed a significantly lower CCR2B gene expression (Figure 2, B and C). To confirm that the increased gene expression for CCR-2 did correspond to an increased protein synthesis, we investigated the protein levels of this receptor in PBMC by Western blotting. As shown in Figure 3, CCR2 protein expression mirrored the changes in CCR2 mRNA abundance observed at the different time points with the two membranes.

Finally, the changes (%) in leukocyte number during HD were taken as an additional parameter of membrane biocompatibility. Leukocyte drop (expressed as percentage of initial white blood cell count) was most pronounced after 15 min of HD, particularly when CA membrane was used (47% of pre-

Figure 2. CCR2 gene expression in PBMC that were isolated from normal subjects (A) and from CA- and EVAL-treated HD patients (B). Freshly isolated PBMC were lysed in guanidinium isothiocyanate, and total RNA was obtained by the single-step method. Monocyte chemoattractant protein-1 (MCP-1) receptor gene expression was investigated by reverse transcription–PCR (RT-PCR) using two different sets of primers that specifically recognize the two splicing variants of CCR2 as described in Materials and Methods. All gels shown are from the same patient and are representative of six healthy subjects and 10 HD patients. Quantification of CCR2/glyceraldehyde-3-phosphate dehydrogenase (GAPDH) ratio is provided in C. *P < 0.001 versus EVAL membrane.

Figure 3. CCR2 protein expression in PBMC that were isolated from normal subjects and from CA- and EVAL-treated patients (A). PBMC were isolated and lysed in RIPA buffer at T0 and T180. CCR2 protein levels were studied by Western blotting using a specific mAb as described in Materials and Methods. All gels shown are from the same patient and are representative of 10 patients. Quantification of CCR2 protein expression is provided in B. *P < 0.001 versus EVAL membrane.
treatment levels), whereas lower leukocyte change was recorded with EVAL membrane (64%; Figure 4). In addition, at the same time, HD monocytopenia was prominent with CA membranes only (data not shown). A return to pretreatment levels was observed by the end of the third and fourth hours of HD.

**Effect of CA and EVAL Membranes on C5b-9 Generation**

The contact between blood constituents and bioincompatible membranes triggers leukocyte activation and migration into the pulmonary capillaries (20,21). Part of these events is thought to be a consequence of dialyzer-associated activation of the complement system through the alternative pathway (21). Recently, the terminal complement complex C5b-9 was suggested to modulate several cell functions in a variety of cell types (22,23). Thus, at the end of each experimental period, we first evaluated the ability of the different membranes to generate C5b-9 complex. At the beginning of HD, a significantly higher level of

**Figure 4.** Leukocyte count during dialysis with CA and EVAL membrane. Whole blood samples were drawn at the indicated time points, and total leukocytes were counted as described in Materials and Methods. *P < 0.01 versus CA.

**Figure 5.** C5b-9 generation during hemodialysis (HD) with CA and EVAL membrane. Plasma concentrations of C5b-9 were measured by ELISA as described in Materials and Methods. The dotted line represents the mean of C5b-9 concentration in the control group (0.16 ± 0.07 μg/ml). *P < 0.02 versus EVAL membrane.

**Figure 6.** Effector protease receptor-1 (EPR-1; A) and protease-activated receptor-2 (PAR-2; C) gene expression in healthy subjects (Control) and uremic patients who underwent HD with either CA or EVAL. Freshly isolated PBMC that were obtained from healthy subjects and HD patients then were lysed in guanidium isothiocyanate. EPR-1 and PAR-2 gene expression was evaluated by RT-PCR and normalized to GAPDH as described in Materials and Methods. All gels shown are from the same patient and are representative of six healthy subjects and 10 HD patients. Quantification of EPR-1/GAPDH and PAR-2/GAPDH ratios are provided in B and D, respectively. *P < 0.001 versus EVAL membrane.
plasma C5b-9 was found in CA-treated (0.22 ± 0.07 µg/ml) as compared with EVAL-treated patients (0.15 ± 0.02 µg/L; P < 0.02; normal value 0.16 ± 0.07 µg/ml). C5b-9 generation was significantly increased at T15 and T180 by both types of membranes and returned to baseline values only in EVAL-treated patients (Figure 5).

Gene Expression of Specific Coagulation Factor Receptors on Normal and Uremic PBMC

Because EVAL has been shown to reduce the activation of the coagulation system, we hypothesized that the improved bio-compatibility of this dialytic membrane may be due to a reduced generation of coagulation factors. Indeed, it is well established that at least two of these serine protease, FXα and thrombin, may modulate mononuclear cell activation through the interaction of several specific cell surface receptors. Thus, we investigated whether the expression levels of these receptors were modulated by the uremic state and/or by dialysis treatment with a specific membrane. The expression of EPR-1, the FXα receptor identified by Altieri et al. (6), was comparable between normal subjects and HD patients at T0 (Figure 6, A and B). However, EPR-1 mRNA abundance was strikingly increased at T180 only in patients who were treated with CA. The prolonged use of EVAL significantly reduced EPR-1 gene expression at T180 (Figure 6, A and B). PAR-2 was highly expressed in normal PBMC (Figure 6, C and D). PAR-2 mRNA levels were increased in PBMC from CA, in particular at T180, whereas in EVAL-treated patients, both basal and T180 gene

**Figure 7.** PAR-2 protein expression in PBMC that were isolated from normal subjects and from CA- and EVAL-treated patients (A). PBMC were isolated and lysed in RIPA buffer at T0 and T180. PAR-2 protein levels were studied by Western blotting using a specific mAb as described in Materials and Methods. All gels shown are from the same patient and are representative of 10 patients. Quantification of PAR-2 protein levels is provided in B. *P < 0.001 versus EVAL membrane.

**Figure 8.** PAR-4 (A) and PAR-1 (C) in healthy subjects (Controls) and uremic patients who underwent HD with either CA or EVAL. Freshly isolated PBMC that were obtained from healthy subjects and HD patients then were lysed in guanidium isothiocyanate. PAR-4 and PAR-1 gene expression was evaluated by RT-PCR and normalized to GAPDH as described in Materials and Methods. All gels shown are from the same patient and are representative of six healthy subjects and 10 HD patients. Quantification of PAR-4/GAPDH and PAR-1/GAPDH ratios are provided in B and D, respectively. *P < 0.001 versus EVAL membrane.
expression was significantly reduced (Figure 6, C and D). Although PAR-2 protein expression at T0 mirrored the mRNA levels, at T180 in CA-treated patients, it was dramatically reduced, suggesting a significant degradation of this receptor very likely as a result of an extensive proteolytic activation (Figure 7). PAR-4, the recently cloned thrombin receptor, was significantly increased only in CA-treated patients (Figure 8, A and B), whereas gene expression of PAR-1, the first identified thrombin receptor, was not modulated by different dialytic membranes or uremic state (Figure 8, C and D).

**In Vitro Study of PBMC Activation**

To define the role of FXa and thrombin in bioincompatibility phenomena, we investigated in vitro the effects of these coagulation factors on CCR-2 gene expression in normal PBMC. FXa, at a concentration of 10 nM, induced a striking increase in CCR2A (data not shown) and CCR2B (Figure 9) gene expression, particularly after 6 and 24 h of incubation, whereas thrombin did not modify the mRNA levels of MCP-1 receptor (data not shown). In addition, FXa caused a significant increase in the levels of cytoplasmic tyrosine-phosphorylated proteins (data not shown) and a striking and time-dependent activation of JNK (Figure 10). It is interesting that inhibition of tyrosine kinases, as well as JNK, by genistein and curcumin, respectively, completely abolished FXa-induced CCR2B gene expression (Figure 11).

**Discussion**

Priming of the coagulation cascade was the first bioincompatibility event to be considered in HD, but its role in the complex inflammatory process primed by the contact between mononuclear cells and dialytic membranes long has been neglected. Even if clinically efficient anticoagulation during a dialysis session is obtained, biochemical markers of platelet and coagulation cascade activation significantly increase during dialysis (2). Hofbauer et al. (24) demonstrated in a group of patients who were undergoing HD with polysulphone the presence of a fibrin network covering a large portion of the fiber surface and protruding into the fiber lumen. This observation suggests also that membranes that are considered highly bio-

![Figure 9](image9.png)

**Figure 9.** Activated factor X (FXa)-induced CCR2 gene expression in normal PBMC (A). freshly isolated PBMC that were obtained from healthy subjects were resuspended in serum-free RPMI at 10⁶/ml and incubated with FXa (10 nM) for the indicated periods. CCR2 gene expression was evaluated by RT-PCR and normalized to GAPDH as described in Materials and Methods. Representative of three independent experiments. Quantification of CCR2/GAPDH ratio is provided in B. *P < 0.001 versus unstimulated.

![Figure 10](image10.png)

**Figure 10.** FXa-induced JNK phosphorylation in normal PBMC (A). Freshly isolated PBMC that were obtained from healthy subjects were resuspended in serum-free RPMI at 10⁶/ml and incubated with FXa (10 nM) for the indicated periods. The cells then were lysed in RIPA buffer. Cellular proteins were separated by PAGE, and JNK phosphorylation was evaluated by Western blotting as described in Materials and Methods. The anti–phospho-JNK blots were stripped and reprobed with mAb that recognize the total form of the enzyme. Representative of three independent experiments. Quantification of phospho-JNK/total JNK ratio is provided in B. *P < 0.001 versus unstimulated.
Activated coagulation factors have a short half-life and are rapidly inactivated. However, thrombin and FXa bound within the fibrin clot may be protected by the effect of their circulating inhibitors (26). Therefore, it is conceivable that the fibrin network that covers the dialysis membrane may represent a reservoir for these proteases. In this study, we demonstrated that uremic PBMC that are exposed to EVAL are less prone to express specific FXa protease receptors. By contrast, the in vivo contact between PBMC and cellulosic membranes is followed by an increased expression of EPR-1 and PAR-2. EPR-1 is necessary to localize FXa in close proximity to the cellular membrane, where it then selectively cleaves and activates PAR-2. The latter behaves as the transducing receptor component, triggering the intracellular signaling pathways (7). The dramatic reduction of PAR-2 protein expression in CA-treated patients in the presence of high mRNA levels suggests that this receptor is degraded extensively during an HD session, most likely as the result of a significant proteolytic activation. Thus, CA-treated uremic patients should be significantly more exposed to the proinflammatory FXa cellular effects. FXa but not thrombin induced a significant increase of JNK phosphorylation and CCR2 gene expression in cultured normal PBMC. This is the first report that a coagulation factor can modulate the expression of the main MCP-1 receptor in circulating mononuclear cells. It is widely known that MCP-1 is a powerful and specific chemotactic and activating factor for monocytes, and we previously reported a striking increase of MCP-1 expression by PBMC during HD (11,12). MCP-1 and CCR2 play a key role in the development of atherosclerosis. Indeed, both MCP-1 and CCR2 knockout mice do not develop atherosclerotic lesions (18,27). Thus, a reduced CCR2 expression may lower the chemotactic response to MCP-1 and likely prevent the recruitment of monocytes into vascular atherosclerotic lesions (18).

It is conceivable that the activation of the coagulation cascade and the subsequent increase in CCR2 expression may be involved in another acute effect of bioincompatible HD: Pulmonary leukosequestration. Leukopenia after blood-membrane contact, as a result of a significant sequestration of leukocytes within the pulmonary capillary, is generally thought to be associated with complement system activation through the release of chemotactic fragments (28,29). This hypothesis would explain the migration and subsequent activation of granulocytes but does not fully account for the monocyte sequestration often observed during bioincompatible treatments (30). In our study, EVAL significantly reduced early monocytopenia when compared with CA treatment, despite a similar generation of C5b-9 in the first 15 min of HD. Considering the striking reduction in CCR2 expression by PBMC in EVAL-treated patients, we hypothesized that this chemokine receptor may play a role in the recruitment of monocytes in the lung microcirculation. This hypothesis assumes that lung microvascular endothelial cells express MCP-1 at a significant level during HD session. There is well-established evidence that HD patients present high circulating levels of a variety of proinflammatory cytokines, including IL-1, TNF-α, and IL-6, that are widely known to induce MCP-1 gene expression in different microvascular endothelial cell lines (1,31,32). Thus, it is conceivable that lung microvascular endothelial cells in these patients secrete a significant amount of MCP-1.

The molecular mechanisms underlying the modulation of CCR2 gene expression in leukocytes still are largely unclear,

**Figure 11.** The effect of tyrosine kinase and JNK inhibition on FXa-induced CCR2 gene expression in normal PBMC (A). Freshly isolated PBMC that were obtained from healthy subjects were resuspended in serum-free RPMI at 10⁶/ml, incubated for 3 h with curcumin or genistein, and then stimulated with FXa (10 nM) for 6 h. The cells were lysed in guanidium isothiocyanate. Representative of three independent experiments. Quantification of CCR-2/GAPDH ratio is provided in B. *P < 0.001 versus unstimulated; **P < 0.001 versus FXa stimulated.
and there are no observations on the signaling pathways and/or transcription factors involved in this event. It is interesting that in our in vitro study, we demonstrated that tyrosine kinase as well as JNK inhibition completely abolished FXa-induced CCR2 gene expression in normal PBMC, suggesting a role for the axis tyrosine kinase-stress-activated kinases in the modulation of this key proinflammatory molecule. The tyrosine kinase signaling module has a pivotal role in the regulation of both the innate and the acquired immune response (33–35). Transgenic mice with a constitutively active HCk, a member of the src family of tyrosine kinases expressed in myelomonocytic cells, spontaneously acquired a lung pathology characterized by extensive mononuclear cell infiltration within the lung parenchyma and alveolar airspaces and around blood vessels (36), a condition that can resemble HD-induced pulmonary leuko-sequestration. It is interesting that src-related cytoplasmic tyrosine kinases are widely known activators of JNK (37). Once activated, JNK, in turn, can phosphorylate jun and facilitate its interaction with fos to form AP-1. This transcription factor then may modulate the gene expression of a variety of proinflammatory cytokines, a hallmark of bioincompatibility (1).

In conclusion, our data support the hypothesis that subclinical clotting activation may cause an increased CCR2 gene expression on uremic PBMC and contribute to HD-related chronic microinflammation. Thus, it is conceivable that the use of a less coagulation activating membrane such as EVAL might improve this condition, reducing specific FXa and MCP-1 cell-surface receptors. In addition, the central role of JNK in the upregulation of CCR2 by FXa may suggest this enzyme as a molecular target for therapeutic intervention in the treatment of HD-related systemic microinflammation. Finally, long-term prospective studies are warranted to confirm the link between the molecular mechanisms identified in our study and the HD-related cardiovascular complications.

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