Bacterial DNA and endothelial damage in haemodialysis patients

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Abstract

Background. An increased percentage of CD14+CD16+ activated monocytes have been reported in peripheral blood from haemodialysis patients. The aim of this study is to investigate if a mild stimulus such as bacterial DNA (CpG-ODNs) contamination may induce an inflammatory response in CD14+CD16+ monocytes from haemodialysis patients and to value the biological consequences of this inflammatory response on endothelial cell damage.

Methods. Circulating mononuclear cells from 20 haemodialysis patients and 15 healthy subjects were studied. CD14+CD16+ and the toll-like receptor 9 (TLR-9) expression were assessed by flow cytometry. Cell culture inserts were used to evaluate the effect of CD14+CD16+ and CpG-ODNs on endothelial cell apoptosis (measured by Tunnel). Intracellular cytokines were measured by Cytometric methods. NF-κB, p38 MAPK, c-Jun PI3K and MEK1/2 activity were modified by specific peptides.

Results. At baseline, CD14+CD16+ have an increased expression of cytoquines and TLR-9. CpG-ODNs caused the production and release of cytoquines in CD14+CD16+, but not in CD14+++ monocytes. This inflammatory response was mediated by intracellular signalling dependent on NF-κB, p38 MARK or c-Jun PI3K but not by MEK1/2 activation. The results of the present study also demonstrate that the inflammatory response induced by the stimulation of CD14+CD16+ by CpG DNA resulted in endothelial cell apoptosis.

Conclusions. The results of the present study demonstrate that in haemodialysis patients there is a subpopulation of pre-activated monocytes that can be stimulated by contaminant bacterial DNA. These activated cells produce and release inflammatory factors that may cause endothelial injury.

Keywords: bacterial DNA; endothelial damage; haemodialysis; inflammation; monocytes

Introduction

Human peripheral blood monocytes from healthy subjects show strong expression of CD14 receptor, and no expression of CD16, so these cells are (CD14++). In some inflammatory diseases, the percentage of peripheral CD14+++ decreases while a subset of monocytes with CD16 expression and more moderate expression of CD14 (CD14+CD16+) is expanded [1,2]. As compared with CD14++, CD14+CD16+ monocytes exhibit more proinflammatory properties, higher TNFα and IL-12 production, lower or absent IL-10 production and higher potency in antigen presentation [3]. Taken together, additional studies suggest that CD14+CD16+ monocytes exhibit characteristics of dendritic cells [4].

Several authors have reported that chronic kidney disease (CKD) patients on regular haemodialysis (HD) present a chronic inflammatory state with monocyte activation that appears to contribute significantly to their cardiovascular disease (CVD), the most important cause of morbidity and mortality in these patients [5,6]. In addition, a large population of these monocytes show a CD14+CD16+ phenotype [7,8]. We have demonstrated that CD14+CD16+ are proinflammatory cells loaded with mature cytokines in the cytoplasm, ready to be secreted [8]. Furthermore, the CD14+CD16+ monocytes from HD patients exhibit shortened telomeres suggesting that these cells have prolonged their life span [8,9]. Taken together these data support the idea that at least in HD patients, CD14+CD16+ monocytes are circulating “primed” monocytes that may play an important role in the perpetuation of chronic inflammation [9]. A variety of factors present in these patients may be involved in monocyte activation including the presence of lipopolysaccharide (LPS) or the use of low biocompatible dialyzers [10,11]. Also, short fragments of bacterial DNA found in the dialysate are newly identified factors that can activate monocytes [12]. Despite the use of ultrapure water, DNA fragments are still present in the dialysate even when bacteriological contamination has been excluded [12]. Bacterial DNA contains immunostimulatory oligodeoxynucleotide (ISS-ODN) sequences of 15–20 base pairs with non-methylated cytosine-guanine (CpG) dinucleotides [13]. CpG dinucleotides can cross the dialyzer membranes through retro-filtration [10]. The CpG
fragments, as well as their synthetic CpG oligodeoxynucleotides (CpG-ODNs), bind to the toll-like receptor 9 (TLR-9) of immunocompetent cells [14] triggering a mitogen-activated protein kinases (MARKs) signalling cascade [15].

The aim of the present study is to investigate if a mild stimulus such as bacterial DNA contamination may induce an inflammatory response in primed CD14+CD16+ monocytes from HD patients. To value the biological consequences of this inflammatory response we set out to define the impact of inflammation on endothelial cell damage.

Material and methods

Human subjects

The 20 subjects included in the study were recruited from the Hemodialysis Unit of Reina Sofia University Hospital (Cordoba, Spain). Blood was obtained from HD patients and from 15 healthy age-matched controls. All patients had been dialyzed using polysulfone membranes (1.8 m², HF80, Fresenius Medical Care, Bad Homburg, Germany), for at least 2 months prior to the initiation of the study. Analysis of the dialysis system always revealed absence of bacteria (<100 Colony forming units per millilitre) or bacteriological contaminant products (endotoxin levels <0.025 endotoxin units) throughout the whole study period. Dialysis efficiency was estimated using eKt/V. All patients, except one, had a native arterio–venous fistula. Medical charts were reviewed to record age, gender, weight, height, underlying renal disease, dialysis vintage and history of transplantation, diabetes, comorbid conditions and current medication. All the patients were treated with darbepoetin to maintain a target of haemoglobin of 11.5–12.5 g/dl. Eighteen patients were treated with 100 µg/week of iron sucrose. The patients were neither on anti-inflammatory nor on immunosuppressive drugs during the 3 months prior to inclusion, not throughout the entire study period. None of the patients had diabetes.

Cell line

Human umbilical vein endothelial cells (HUVEC Clonetics, San Diego, CA, USA) were cultured in a M199 medium and supplemented with 20% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 20 mmol/l l-glutamine at 37°C and 5% CO₂. All cell culture media were purchased from GIBCO BRL (Grand Island, NY, USA).

Cell preparation and culture of human mononuclear cells

Circulating human mononuclear cells were obtained from whole blood. Buffy coat cells were separated by a differential centrifugation gradient (Ficoll/Hypaque, Pharmacia LKB, Uppsala, Sweden). Thereafter, cells were seeded on 24-well culture plates (Falcon, Becton Dickinson and Company, Paramus, NJ, USA) in a complete culture medium that contained RPMI 1640 supplemented with l-glutamine (2 mM), Hepes (20 mM), sodium pyruvate (1 mM), streptomycin (50 ng/ml), penicillin (100 U/ml) and 10% fetal calf serum (FCS) at 37°C in 5% CO₂/95% air atmosphere (medium and additives were supplied by Bio-Whittaker, Walkersville, MD, USA). FCS was heated for 1 h at 56°C to eliminate the complement-activating fractions.

Study of inflammation-induced endothelial cell apoptosis using cell culture inserts

Insert preparation. Cell culture inserts (3.0 µm) (Falcon-Becton Dickinson, San José, CA, USA) for use in 24-well tissue culture plates were coated with 70 µg/ml type I collagen in 20 mM acetic acid for 1 h at 23°C. The inserts were then washed with HEPES buffered saline (137 mM NaCl–4 mM KCl–6 mM glucose–20 mM HEPES, pH 7.45) to remove excess protein. The complete medium was added to both the insert and the well, and it was left to equilibrate for 3 h at 37°C in 5% CO₂-air prior to use.

Cell culture. Mononuclear cells (1 x 10⁵/ml) were seeded on 24-well tissue culture plates in a complete culture medium. HUVEC cells were trypsinized from tissue culture flasks, washed three times with the complete medium and seeded on the upper side of 2 x 10⁵ cells/insert that were placed into each well in direct contact with the medium cultivating the mononuclear cells.

After a given culture period (see the Results section), the inserts containing HUVEC cells were removed from the culture plates and adherent cells were detached using a scrape. These cells were then centrifuged and resuspended in PBS.

All cell culture media were purchased from GIBCO BRL (Grand Island, NY, USA).

Reagents

Oligodeoxynucleotides (ODNs) (10 µg/ml) were synthesized by Invitrogen Life Technologies, OR, USA, as follows: PS-ODN 2006 T-C-G-T-C-G-T-T-T-G-T-C-G-T-T-T-G-T-G-C-G-T-T and PO-ODN 2006b T-C-C-C-T-C-T-T-T-T-G-T-C-C-T-G for all experiments, a dose of 0.5 µM CpG-ODN fragments was used.

LPS Escherichia coli O55:B5 was purchased from Calbiochem, La Jolla, CA, USA.

NF-κB activity is the main intracellular pathway mediating inflammatory response in immunocompetent cells stimulated by CpG-ODN. The following protein and chemical reagents were used to modulate kinases or NF-κB-dependent pathways that may be involved in cell activation and/or apoptosis induced by CpG-ODN: SN50, a cell-permeable peptide specifically to inhibit nuclear translocation of the NF-κB complex and the control inactive peptide SN50M purchased from Calbiochem, La Jolla, CA, USA. Both peptides were endotoxin free, as certified by the manufacturer. U0126, [1-(4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)-butadiene], a selective MEK inhibitor, 5 µM; SP600125, anthra[1, 9-cd]pyrazol-6(2H)-one1, 9-pyrazoloanthrone, a selective c-Jun N-terminal kinase (JNK) inhibitor; SB239063, trans-1-(4-hydroxy cyclohexyl)-4-(fluorophenyl)-5-(2-methoxy pyrimidin-4-yl)imidazole, a p38 MAPK
inhibitor. All these inhibitors were purchased from Calbiochem, La Jolla, CA, USA.

**Cell fluorescence**

To identify CD14+CD16+ monocytes, cells (10^5/ml) were incubated for 30 min at 4°C with the monoclonal antibodies (mAb) M5E2 against the molecule CD14 conjugated with peridinin chlorophyll protein (PerCP) or 3G8 against the molecule CD16 conjugated with phycoerythrin (PE). Both anti-CD14 and anti-CD16 mAb and the appropriate isotype controls for each antibody were provided by Becton Dickinson, San José, CA, USA.

Cytofluorometric analysis was performed using a FACScan cytometer (Becton Dickinson, San José, CA, USA). After incubation, cells were washed and resuspended in 0.5 ml 1% formaldehyde and stored at 4°C for flow cytometric analysis. Monocytes were identified by forward and side light scatter properties. Ten thousand cells were analysed per sample. The percentage of CD14+CD16− and CD14+CD16+ monocytes was determined after subtraction of nonspecific staining, as identified by the isotype control histogram.

To study TLR-9 expression, cells stained with surface mAbs anti-CD14-PerCP and anti-CD16-PE were simultaneously labelled with an anti-TLR-9 Fluorescein Isothiocyanate (FITC) conjugated antibody (eBiosciences, San Diego, CA, USA).

**Measurement of intracellular cytokines**

Cytokines expression was studied in isolated monocytes cultured for 6 h in the presence of brefeldin A (BFA), with and without LPS. For these studies, 600 µl samples were incubated for 6 h at 37°C in 15 ml polypropylene tubes (Nunc A/S, Denmark) in the presence of BFA at 2 µg/ml (Sigma, St Louis, MO, USA). The samples were left untreated or stimulated with LPS from E. coli (Sigma, Chemical Co. Poole, UK) or CpG-DNA (Calbiochem, La Jolla, CA, USA). After incubation, cells (10^5/ml) were washed and stained with surface mAbs anti-CD14-PerCP and anti-CD16-PE. Thereafter, cells were washed and permeabilized using FACS permeabilizing solution (Becton Dickinson, San José, CA, USA) and incubated with the appropriate FITC conjugated antibody: anti-IL-6 and anti-TNFα (Becton Dickinson, San José, CA, USA) or the corresponding isotype control. After incubation, cells were washed and resuspended in 0.5 ml 1% formaldehyde until flow cytometric analysis.

**Cytometric bead array immunoassay**

The cytometric bead array technique was based on the binding between microparticles labelled with different intensities of fluorescence and antibodies. The maximum wavelength emission was ~650 nm (fluorescence-3 channel, FL-3). The particles were bound by a covalent bond to an antibody against one of the cytokines IFN-γ or IL-10 (PharMingen, San Diego, CA, USA). This method allowed for the simultaneous determination of different cytokines in the same sample. The cytokines were captured directly in the immunoassay using different antibodies bound to PE, which emitted at 585 nm (fluorescence-2 channel, FL-2). The PE-conjugated detector antibody completed the sandwich. The intensity measured in FL-2 was proportional to the concentration of cytokines in the sample, which was quantified from a calibration curve. An important characteristic of the assay system was that the calibrators, the antibody-bead reagent, and the second antibody reagent, antibody-PE, were made from mixtures of cytokines. Standard curves (0–5000 pg/ml) were derived from a set of calibrators and the same set was used for all assays. Then, 50 µl of either the sample or cytokine standard was added to the mixture of 50 µl of antibody-PE detector and 50 µl of antibody-bead reagent. The mixture (150 µl) was incubated for 160 min in the dark at room temperature and washed. The data were acquired using the flow cytometer.

**TUNEL assay**

Apoptosis was measured using a kit that was based on the terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine 5'-triphosphate (dUTP) nick-end labelling (TUNEL) (Roche Diagnostics Boehringer Mannheim, Mannheim, Germany). In accordance with the manufacturer’s instructions, 10^6 cells were fixed with 4% paraformaldehyde for 30 min at room temperature, washed and permeabilized for 2 min in ice with 0.1% Triton X-100. After washing, cells were decanted and resuspended in 50 µl of TUNEL reaction mixture (5 µl of TUNEL enzyme containing TdT mixed with 45 µl of TUNEL label containing PE-dUTP and dNTP nucleotides) or in 50 µl of TUNEL label, which served as the negative control. After 60 min at 37°C in a humid atmosphere, cells were washed three times in a wash buffer (PBS + 0.1% NaN₃ + 10% autologous serum) and analysed by flow cytometry.

**Statistical analyses**

Results are expressed as means ± SD. Non-parametric data were compared using the Kruskal–Wallis test. The Mann–Whitney U-test for unpaired data and the Wilcoxon signed-rank test for paired data were used to compare means. Differences were regarded as significant at P < 0.05.

**Results**

The demographic and main biochemical parameters of HD patients are shown in Table 1.

CD14+/CD16+ inflammatory cells are increased in patients in haemodialysis

As observed in other studies, HD patients present two populations of monocytes with different expressions of CD14 and CD16 antigens (Figure 1). One subset of monocyte cells exhibits strong CD14 expression with no CD16 expression (CD14++). Other cells have moderate expression of both CD14 and CD16 (CD14+CD16+). There are also monocytes ranging from CD14++ to CD14+CD16+ that, in this study, have been excluded from further analysis.
As expected, a high percentage of CD14+CD16+ cells from patients contain a high cytoplasmic level of IL-6 and TNF (76 ± 11% and 83 ± 9% of cells were positive for IL-6 and TNF, respectively), measured by flow cytometry using specific mAb against mature intracellular cytokines. In contrast, only a low percentage of CD14++ cells (<5%) showed detectable levels of cytoplasmic cytokines.

**TLR-9 expression of CD14+CD16+ cells from patients**

The small number of CD14+CD16+ cells in control subjects did not allow us to perform biochemical and functional studies. Thus, functional differences between CD14++ and CD14+CD16+ monocytes were performed only on cells from HD patients. The TLR-9 receptor is expressed in both CD14++ and CD14+CD16+ monocytes. Expression of TLR-9 receptor was found in 89 ± 6% of CD14++ monocytes and 91 ± 4% of CD14+CD16+ monocytes. However, as shown in a representative histogram (Figure 2), the density of TLR-9 receptor expression (measured according to the mean fluorescence channel) was higher in CD14+CD16+ (426 ± 51) than CD14++ monocytes (323 ± 37) (P = 0.02).

**CpG-ODN effect in cytokines production by CD14+CD16+ cells from patients**

After culture with sub-stimulating doses of CpG-ODN, the percentage of CD14++ or CD14+CD16+ monocytes expressing intracellular cytokines did not change significantly. However, after stimulation with CpG-ODN the amount of intracellular cytokines per cell increased in both subsets of monocytes. Thus, in CD14+CD16+ monocytes the mean fluorescence channel (MFC) (amount of cytokine per cell) increased from 293 ± 61 to 391 ± 59 for IL-6 (P = 0.005), and from 331 ± 52 to 487 ± 52 for TNFα (P = 0.008). In CD14++ monocytes, the MFC for IL-6 increased from 278 ± 57 to 354 ± 63 (P = 0.01), and for TNFα from 316 ± 67 to 432 ± 74 (P = 0.04) (Figure 3).

Cytokines are released into the culture medium by activated monocytes from HD patients. Soluble IL-6 (18.1 ± 7.2 ng/ml) and TNFα (43.5 ± 12.4 ng/ml) were detected in the cell culture supernatant of monocytes from HD patients. However, cytokines were not detectable in the supernatant of cultured monocytes from healthy subjects. After the addition of sub-stimulating doses of CpG-ODN, increased secretion of cytokine was observed in monocytes from HD patients (69.6 ± 12.3 P = 0.001, and 163.3 ± 18.9 ng/ml P = 0.004, for IL-6 and TNFα, respectively), whereas soluble cytokines were undetectable when monocytes from healthy subjects were cultured with sub-stimulating doses of CpG-ODN.

**The role of the NF-κB pathway and kinase activity in inflammation induced by CpG-ODN fragments**

NF-κB activity is the main intracellular pathway mediating inflammatory response in immunocompetent cells stimulated by CpG-ODN. Therefore, we explored the role of

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**Table 1. Characteristics and laboratory values of the haemodialysis patients**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Haemodialysis</th>
</tr>
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<tbody>
<tr>
<td>Number of patients</td>
<td>20</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>12/8</td>
</tr>
<tr>
<td>Age (years; mean, minimum–maximum)</td>
<td>54.8 (23–79)</td>
</tr>
<tr>
<td>Dialysis vintage (months; mean, minimum–maximum)</td>
<td>55.1 (15–130)</td>
</tr>
<tr>
<td>Duration of dialysis treatment (minutes; mean, minimum–maximum)</td>
<td>221.3 (180–270)</td>
</tr>
<tr>
<td>Leucocytes (mean, minimum–maximum)</td>
<td>7856.7 (4612–10526)</td>
</tr>
<tr>
<td>Neutrophils (mean, minimum–maximum)</td>
<td>4820 (2150–8340)</td>
</tr>
<tr>
<td>Lymphocytes (mean, minimum–maximum)</td>
<td>2350 (1904–3256)</td>
</tr>
<tr>
<td>Monocytes (mean, minimum–maximum)</td>
<td>390 (129–465)</td>
</tr>
<tr>
<td>Eosinophils (mean, minimum–maximum)</td>
<td>130 (11–278)</td>
</tr>
<tr>
<td>Basophils (mean, minimum–maximum)</td>
<td>92 (53–129)</td>
</tr>
<tr>
<td>Haemoglobin (g/dl; mean, minimum–maximum)</td>
<td>12.25 (9.2–14.3)</td>
</tr>
<tr>
<td>Ferritin (mcg/ml; mean, minimum–maximum)</td>
<td>512 (186–1130)</td>
</tr>
<tr>
<td>Albumin (g/dl; mean, minimum–maximum)</td>
<td>4.1 (3.7–4.7)</td>
</tr>
<tr>
<td>CRP (mg/l; median, minimum–maximum)</td>
<td>7.02 (0.9–23.4)</td>
</tr>
<tr>
<td>Beta2-microglobulin (mean, minimum–maximum)</td>
<td>25.9 (16.2–29.6)</td>
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*Fig. 1. Expression of CD14+CD16+ cells in haemodialysis patients. Representative flow cytometry of the CD14+CD16+ cell population of monocytes from HD patients. Monocytes have been selected on a forward scatter/side scatter dot plot histogram. Peridinin chlorophyll protein (PerCP)-staining CD14 and phycoerythrin (PE)-staining CD16 of the gated monocyte population CD14++ monocytes in gate 1 and CD14+CD16+ monocytes in gate 2. The cell distribution according to the expression of CD14+ and CD16+ shows two different subsets of cells. We have gated one subset exhibiting strong CD14 expression with no CD16 expression (CD14++). The second gate (CD14+CD16+) defines a subset of cells with moderate expression of both CD14 and CD16 (16.4% of monocytes in this sample).*

because these ‘transitional’ cells do not belong to either of the two subsets above mentioned.

The percentage of CD14++ and CD14+CD16+ monocytes from HD patients was 81.7 ± 7.2 and 18.3 ± 5.4, respectively. In contrast, in healthy subjects the percentage of CD14++ and CD14+CD16+ monocytes was 92.3 ± 6.7 and 8.1 ± 3.2 (P = 0.005 and P = 0.001), respectively, versus CD14++ and CD14+CD16+ monocytes from HD patients.
Endothelial damage in haemodialysis patients

Fig. 2. TLR-9 expression in monocytes from haemodialysis patients. Representative histograms of TLR-9 expression in both subset CD14++ (A) and CD14+CD16+ (B). Triple fluorescence was carried using FITC-conjugated TLR-9, and simultaneous labelling with PerCP-conjugated CD14 and PE-conjugated CD16. CD14++ and CD14+CD16+ subsets were selected as indicated in Figure 1. TLR-9 positive cells are calculated as the expression relative to isotype staining for both monocyte subpopulations (white curve).

Fig. 3. CpG-ODN affects cytokines production by CD14+CD16+ cells. The intracytoplasmic expression of cytokines in both monocytes cells from haemodialysis patients was calculated in both CD14++ and CD14+CD16+ subsets after stimulation with synthetic CpG oligodeoxynucleotides (CpG-ODN) (0.50 µM for 24 h of culture). The values are units of intensity of fluorescence relative to the mean fluorescence channel (MFC). (A) IL-6 expression was significantly increased after CpG-ODN stimulation ($P = 0.005$). (B) Similarly, TNFα expression was significantly increased after treatment with CpG-ODN ($P = 0.008$).

Monocytes from patients stimulated with CpG-ODN-secreted factors that induce apoptosis in endothelial cells

Mononuclear cells and endothelial cells were co-cultured in the same well but separated by a membrane of 0.4-µm pore size (insert system). Under these culture conditions, endothelial cells were exposed to the cytokines and other factors released in situ by co-cultured monocytes. CpG-ODN did not induce apoptosis in either endothelial cells cultured without monocytes or endothelial cells co-cultured with monocytes from healthy subjects (data not shown). However, apoptosis (37.9 ± 7.6%) was found in endothelial cells co-cultured with monocytes from HD patients and CpG-ODN.

Specific inhibitors used to test the role of p38 MAPK-, c-Jun NH2-terminal kinase- and MEK (MEK1/2)-dependent signals in the inflammatory response were induced by CpG-ODN in monocytes from HD patients. Figure 4B shows that there was no release of cytokines when monocytes from HD patients were cultured in the presence of p38 MARK and c-Jun inhibitors. However, the inhibition of signals that were dependent on the MEK pathway did not affect CpG-ODN-mediated cytokines induction.

Monocytes from patients stimulated with CpG-ODN-secreted factors that induce apoptosis in endothelial cells
Fig. 4. The role of the NF-κB pathway and kinase activity in inflammation induced by CpG-ODN fragments. (A) The role of the inhibition of NF-κB signalling (using SN50) in CD14+CD16+ monocytes from HD patients activated by CpG-ODN. Release of IL-6 or TNFα cytokines to the culture medium by monocytes from HD patients was not observed in the presence of SN50 peptide (P < 0.01 versus CpG-ODN stimulated culture). SN50M was used as inactive peptide control. (B) Specific inhibitors were used to test the role of p38 MAPK-, c-Jun NH2-terminal kinase- and MEK (MEK1/2)-dependent signals. Only p38 MARK and c-Jun inhibitors blocked the release of IL-6 or TNFα cytokines in cultured to monocytes from HD patients stimulated with CpG-ODN (P < 0.01 from IL-6 and TNFα).

Table 2. Role of NF-κB and P38 MAPK in the apoptosis and intracellular cytokine levels of endothelial cells co-cultured with mononuclear cells from HD patients stimulated with CpG-ODN

<table>
<thead>
<tr>
<th>Cytokines (MFC)</th>
<th>Apoptosis</th>
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<tbody>
<tr>
<td>Basal</td>
<td>IL-6</td>
</tr>
<tr>
<td>NF-κB</td>
<td>391 ± 59</td>
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<tr>
<td>P38 MAPK</td>
<td>229 ± 36*</td>
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<td></td>
<td>301 ± 45*</td>
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</table>

All data are expressed as means ± SD from 20 patients studied. MFC: mean fluorescence channel.

*P < 0.01 versus Basal.

Discussion

CD14+CD16+ monocytes are a subset of monocytes that are expanded in the peripheral blood of patients with systemic inflammatory diseases including patients on HD and patients with sepsis or HIV infection [5–8]. In addition, in a previous study we have reported that DNAβ fragments were present in theoretically sterile dialysis fluid and that DNAβ fragments may contribute to the prolonged life span of inflammatory cells from CKD patients on regular HD [16]. In the present study we have shown that CD14+CD16+ monocytes are activated cells that produce and release inflammatory cytokines in response to the sub-stimulating concentration of bacterial DNA, and may induce endothelial cell damage. These data support a role for CD14+CD16+ monocytes not only in the induction and perpetuation of chronic inflammatory diseases but also in CVDs that have more patients with chronic inflammatory diseases.

As described earlier, we found a high percentage of CD14+CD16+ cells in the peripheral blood of HD patients [8,9]. The increase in the percentage of CD14+CD16+ cells was associated with a decrease in CD14++ monocytes. This finding supports the thesis that both populations may share a common origin. Furthermore, CD14+CD16+ and CD14++ cells may be the same cell population at different maturation stages. Furthermore, we observed that CD14+CD16+ monocytes from HD patients show intracellular mature cytokines, reinforcing the notion that they are activated monocytes with proinflammatory activity [8].

In the present study we have observed that CD14+CD16+ monocytes from HD patients have a high density of TLR-9 expression. Previous studies demonstrated that sepsis-related proteins might modulate the expression of TLR-2 and TLR-4 in CD14+CD16+ monocytes [17]. In addition, it has been demonstrated that CD14+CD16+ monocytes are the main TNFα producers after stimulation with LPS [18]. It is reasonable to think that the high expression of TLR-4 and TLR-9
receptors in CD14+CD16+ monocytes enables these cells to be stimulated by bacterial and virus products that interact with these receptors, and this may explain our observation that sub-stimulating doses of bacterial DNA resulted in increased production and release of inflammatory cytokines by CD14+CD16+ monocytes from HD patients. Additionally, these results support our previous data suggesting that CD14+CD16+ monocytes play an important role in the inflammatory process at least in CKD patients [8,19].

MARKs-dependent signalling modulates the inflammatory response in immunocompetent monocyte cells stimulated with CpG-ODNs. However, there are differences in the kinases stimulated by CpG-ODNs in macrophages and other immunocompetent cells such as dendritic cells [20]. On the other hand, CD14+CD16+ monocytes are activated monocytes that share the functional response with dendritic cells. In this study we evaluated some intracellular pathways activated in CD14+CD16+ monocytes. The results obtained indicate that the inflammatory activity induced by bacterial DNA on CD14+CD16+ monocytes is mediated by MARKs. These results are relevant, since specific inhibitors for some of these pathways, such as the P38 MAPK pathway, are being proposed as a therapy for chronic inflammatory pathologies [20–22]. Interestingly, cellular activation induced by bacterial DNA was not inhibited by the presence of selective inhibitors of MEK1/2 activity. It has been demonstrated that ERK pathway plays a relevant role in bacterial DNA-induced inflammatory activation in macrophages, but not in dendritic cells [20]. The latter finding supports the notion that CD14+CD16+ cells behave functionally like dendritic cells, and therefore they have a high capacity to induce allogenic immune responses [23,24].

There is a link between inflammation with endothelial damage and CVD in HD patients [16,25]. In this study we observed that in response to sub-stimulating doses of bacterial DNA, activated monocytes released inflammatory mediators that induce endothelial cells apoptosis. These results suggest that the increased inflammatory activity produced by CD14+CD16+ monocytes in HD patients may negatively affect the cardiovascular system of these patients. Supporting this hypothesis, agents that inhibit soluble cytokines production, such as p38 MAPKs and NF-κB also inhibit endothelial cell apoptosis.

Our results confirm in CKD-HD patients the data previously reported in individuals with chronic immune activation [26] and in murine macrophages [27]. The results of the present study support that CD14+CD16+ monocytes are pre-activated cells, that produce and release inflammatory factors in response to sub-stimulating doses of ubiquitous contaminants agents such as the bacterial DNA. In addition, this study also demonstrate that inflammation maintained by CD14+CD16+ monocytes induce endothelial cell damage, a result that may be relevant because endothelial cell damage is the first step in atherosclerosis and CVD.

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