Impaired antiviral activity of monocytes from patients on hemodiafiltration

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ABSTRACT

Background: The aim of our study was to determine whether intermittent hemodiafiltration (HDF) leads to an alteration in monocyte antiviral activity as well as in the in vitro release of cytokines such as interleukin-12 (IL-12), tumor necrosis factor-α (TNF-α) and interferon-α (IFN-α) by the same cells.

Methods: We enrolled 25 patients undergoing HDF for 3.5-4 hours 3 times a week (12 men, 13 women; mean age 58 ± 6.7 years) and 25 healthy donors (ND) (12 men, 13 women; mean age 57 ± 8 years). Monocytes from peripheral blood mononuclear cells were isolated with a Monocyte Isolation Kit II. Monocytic cells were infected with herpes simplex virus type 2 (HSV-2). Cytokines were assayed in supernatants.

Results: The in vitro antiviral activity of monocytes from HDF patients was significantly impaired with respect to ND. Furthermore, monocytes from post-HDF patients were more prone to viral infection. Lipopolysaccharide (LPS) stimulation induced significant viral inhibition only in monocytes from NDs (p<0.05). The cytokine pattern (TNF-α, IFN-α and IL-12) in monocytes stimulated with LPS was markedly inhibited in HDF patients compared with ND (p<0.05). A basal production of TNF-α was found in monocytes from pre-HDF and post-HDF patients. No IFN-α production was found in LPS-stimulated and HSV-2-infected monocytes from pre-HDF and post-HDF patients. IL-12 production appeared significantly decreased after HDF in all experimental conditions (p<0.05).

Conclusions: The significant increase of viral replication in monocytes from HDF patients compared with healthy donors could be related to a significant reduction of cytokine production. Moreover, the dialytic session influenced the intrinsic antiviral activity of monocytes, favoring viral replication.

Key words: Cytokines, Hemodiafiltration, Herpesvirus, Monocytes

INTRODUCTION

During end-stage renal disease (ESRD), the immune system is severely affected. There is a great deal of scientific evidence demonstrating that the immune dysregulation in such patients can be related to a severe impairment of cell-mediated immunity, as well as to lymphocytopenia (1, 2). Immunological alterations associated with chronic hemodialysis may be explained by shortened lymphocyte survival, lymphopenia and enhanced suppressor T-cell activity, leading to altered tumor surveillance and impaired immune response to infectious agents such as viruses. In fact, many of the complications experienced by patients undergoing hemodialysis can be attributed to their altered immune defense mechanisms (3). Moreover, it is well documented that alteration of the pattern of cytokine response in such patients plays an important regulatory role in their antiviral defenses (4). A complex network of cytokines and chemokines with pleiotropic effects orchestrates the immune response of the host to several viruses (5). Defined effector mechanisms include the release of cytokines such as interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α) and other types of interferons such as interferon-α (IFN-α), with wide-ranging antiviral effects. These immunoregulatory cytokines may induce an antiviral state in the cells (e.g. via IFN-γ) or destroy virus-infected cells (i.e., via TNF-α). Furthermore, they may stimulate cytotoxicity and cytokine production by T cells and natural killer cells, and may initiate the development of Th1 cells (i.e., via IFN-γ, IL-12 and IL-18) (6-8). Herpes simplex viruses (HSV) establish lifelong, latent infections in the peripheral nervous system, so escaping the immune response and persisting in the host (9, 10). Thus, the outcome of infection can be strongly influenced from the pattern of cytokines induced by the virus.

The aim of this study was to investigate in vitro intrinsic antiviral activity against herpes simplex virus type 2 (HSV-2) of monocytes obtained from patients before and after high-effi-
ciency hemodiafiltration (HDF). This kind of substitutive treatment shows a higher biocompatibility and lower mortality rates than conventional hemodialysis (11, 12), besides greater dialytic efficiency due to both diffusive and convective mechanisms (13).

Finally, the production of cytokines such as TNF-α, IFN-α and IL-12 involved in antiviral immune response was analyzed in supernatants of monocyte cultures in different conditions.

**Subjects and Methods**

**Patients**

We investigated 50 subjects: 25 patients on regular maintenance HDF with acetate-free biofiltration (AFB; Integra Physiol Hospal, Bologna, Italy) with polyacrylonitrile filters (AN69-ST; Bologna Hospal, Italy) for 3.5-4 hours 3 times a week (13 women, 12 men; mean age 58 ± 6.7 years, mean dialytic age 2.7 ± 1.6 years; residual glomerular filtration rate 2.3 ± 0.6 ml/min) and 25 age-matched healthy volunteers (13 women, 12 men; mean age 57 ± 8 years) without any evidence in their clinical history and clinical examination of infections, diabetes or drug treatments (Tabs. I and II).

The patients were on dialysis for primary interstitial nephritis (n=3), nephrovascular disease (n=5), diabetic nephropathy (n=5), polycystic kidney disease (n=2) or glomerulonephritis (n=10); all those on dialysis were given intravenous recombinant human erythropoietin 3 times a week (mean dosage 85.7 ± 11.2 IU/kg body weight per week). Kt/V, estimated following the Daugirdas equation, was maintained between 1.2 and 1.3 (Tab. I), and the normalized protein catabolic rate equation was from 1.0 to 1.3g/kg per day. Patients affected by hepatitis B or C were excluded from the study. Both control subjects and patients were nonsmokers. The local ethics committee approved the study, and informed consent was obtained from all subjects. Of the 25 patients on HDF, only 3 had tumors: 2 had parathyroid adenomas (diagnoses made 6 and 8 months, respectively, after the onset of uremia); 1 patient had papillary carcinoma of thyroid gland (diagnosed 3 months after the onset of uremia).

One blood sample was drawn from the cubital vein of each subject. In HDF patients, 2 samples were drawn: predialytic (pre-HDF, 5 minutes before starting dialysis), postdialytic (post-HDF, 5 minutes after the end of the session). Throughout the study, each patient complied with fluid and dietary restrictions, and maintained a constant ultrafiltration volume.

**Hemodialysis technique**

All of the patients were regularly treated with HDF. The dialysis bath was contained in 1 bag (SAFEBAG 93G; Hospal Spa, Italy), and was composed of NaCl (284.31 g/L), KCl (5.22 g/dL), CaCl (10.29 g/L), MgCl2 (2.63 g/L) and glucose (35 g/L). Blood flow was 300 ml/min, bicarbonate infusion 2,000 ml/hour (with BIOSOL sacks AFB 145; Hospal) and the dialysis membrane was AN69. This technique is also more effective than traditional dialysis in guaranteeing a greater cardiovascular tolerance as with hydric subtraction, and is therefore more appropriate for the treatment of patients with hemodynamic instability.

**Table I**

<table>
<thead>
<tr>
<th>Variables</th>
<th>HDF patients (n=25)</th>
<th>Healthy subjects (n=25)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>58 ± 6.7</td>
<td>57 ± 8</td>
</tr>
<tr>
<td>Sex, M/F</td>
<td>12/13</td>
<td>12/13</td>
</tr>
<tr>
<td>Duration of dialysis, years</td>
<td>2.70 ± 1.60</td>
<td>-</td>
</tr>
<tr>
<td>Hemoglobin, g/dL</td>
<td>11.4 ± 1.44</td>
<td>14.3 ± 2.12</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>36.3 ± 4.94</td>
<td>44.9 ± 2.50</td>
</tr>
<tr>
<td>Albumin, g/dL</td>
<td>3.70 ± 0.50</td>
<td>4.70 ± 0.20</td>
</tr>
<tr>
<td>HD dose, Kt/V</td>
<td>1.30 ± 0.28</td>
<td>-</td>
</tr>
</tbody>
</table>

Values are means ± SD.
HD = hemodialysis; HDF = hemodiafiltration; Kt/V = Daugirdas calculation of dialysis urea clearance normalized for body size.
Isolation of human monocytes

Peripheral venous blood was taken from HDF patients and control subjects via a 20-gauge butterfly inserted into a forearm vein. Peripheral blood mononuclear cells (PBMCs) were isolated from freshly collected heparinized venous blood after centrifugation with a Histopaque gradient (Sigma-Aldrich, Milan, Italy) (14). Venous blood of ESRD patients was collected 5 minutes before starting dialysis (pre-HDF) and 5 minutes after the end of the session (post-HDF). To isolate monocytes from PBMCs, Monocyte Isolation Kit II, (Miltenyi Biotec, Bologna, Italy) was used. The Monocyte Isolation Kit II is an indirect magnetic labeling system for the isolation of monocytes from PBMCs. Briefly, non-monocytes – i.e., T cells, NK cells, B cells, dendritic cells and basophils – were magnetically labeled using a cocktail of monoclonal antibodies against CD3, CD7, CD16, CD19, CD56, CD123 and glycophorin A, and antibiotin Microbeads. Isolation of pure monocytes was achieved by depletion of the magnetically labeled cells.

Monocytes were cultured at 37°C in a 5% CO2 atmosphere at a concentration of 2.5x10^5 cells/mL per well in RPMI 1640 medium supplemented with 50 µg/mL gentamicin and 10% heat-inactivated fetal calf serum (FCS), 1 mM pyruvate, 1 mM non-essential amino acids, 1 mM HEPES and 50 µM 2-mercaptoethanol (all reagents from Sigma-Aldrich, Milan, Italy). To determine the effect of different incubation times on cells viability, a cytotoxicity test was used as reported below.

Lipopolysaccharide (LPS) from Escherichia coli strain 055:B5 was used as positive control. Twenty-four hours posttreatment, the supernatants were harvested, aliquoted and stored at –80°C until cytokine analysis.

Monocyte infection

In a second series of experiments, the cells were infected with HSV-2 at multiplicity of infection (MOI) 0.1 for 24 hours at 37°C in 5% CO2, then the plates were frozen and thawed 3 times to release the intracellular virus. Cell lysates and supernatants were kept at –80°C until virus titration.

Virus

HSV-2 was used throughout the study. HSV-2 infection was propagated on WISH cell lines. Viral stocks were prepared by pelleting infected cells exhibiting cytopathic effect, and freezing aliquots at -80°C. The virus titer was assessed on WISH cells using Dulbecco’s method. Briefly, the cultures were frozen 24 hours after infection at -70°C, and, after thawing, the total amount of virus present in the medium and associated with the cells was determined by a plaque assay. The virus was titrated in duplicate by infecting WISH cell monolayers with sequential 10-fold dilutions of the virus preparations. Medium containing 10% of FCS was added after a 1-hour adsorption period, and the cells were incubated for 48 hours. The medium was removed, the cells were stained with 1% methylene blue in distilled water, and the plaques were scored and expressed as number of plaque-forming units per milliliter (PFU/mL).

Cytotoxicity test

To determine the effect of different incubation times on cell viability, a colorimetric assay was used as described in the following table:

<table>
<thead>
<tr>
<th>TABLE II</th>
<th>CHARACTERISTICS OF HD PATIENTS BEFORE AND AFTER AN HDF SESSION AND CONTROL HEALTHY GROUP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before dialysis</td>
</tr>
<tr>
<td>Creatinine, mg/dL</td>
<td>8.44 ± 2.01</td>
</tr>
<tr>
<td>Urea, mg/dL</td>
<td>130 ± 25.1</td>
</tr>
<tr>
<td>Systolic blood pressure, mm HG</td>
<td>138 ± 20.3</td>
</tr>
<tr>
<td>Diastolic blood pressure, mm HG</td>
<td>70.0 ± 2.30</td>
</tr>
<tr>
<td>Leukocytes, cells/mm³</td>
<td>7,231 ± 2,813</td>
</tr>
<tr>
<td>Lymphocytes, cells/mm³</td>
<td>1,590 ± 822.2</td>
</tr>
<tr>
<td>Monocytes (cells/mm³)</td>
<td>636.6 ± 312.2</td>
</tr>
</tbody>
</table>

Values are means ± SD.
by Mosmann (15). The assay is based on the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium-bromide (MTT) (Sigma, Milan, Italy). This pale yellow substrate is cleaved by active mitochondria to produce a dark blue formazan product. Briefly, monocytes were seeded in 96-microwell plates at 2×10^4 per well, then MTT diluted in saline solution was added to the cells and incubated for 4 hours. Then acid propan-2-ol (0.04M HCl in propan-2-ol) (Sigma, Milan, Italy) was used to solubilize the formed crystals. The plates were read with a MicroELISA reader using a wavelength of 570 nm. Cytotoxicity percentage was calculated as follows: 1 - [(experiment OD - lysis control OD) / (cell control OD - lysis control OD)] × 100; where OD is the optical density at 570 nm.

**Limulus test**

Culture media and reagents tested for the presence of endotoxin by E-Toxate kit (Sigma, Milan, Italy) were found to contain ≤10 pg of endotoxin per milliliter.

**Cytokine evaluations**

Supernatants from human monocytes, in different experimental conditions, were collected and assayed for the presence of IFN-α, TNF-α and IL-12 by an immunoenzymatic method (ELISA): human IFN-α immunoassay, human TNF-α immunoassay and human IL-12 immunoassay, all from Bender Medsystems (Milan, Italy). The limit of detection of the assay was 4.8 pg/mL for IFN-α, 5.8 pg/mL for TNF-α and 19.1 pg/mL for IL-12.

**Statistical evaluation**

Results are expressed as means ± standard deviation. Data were analyzed by 1-way analysis of variance (ANOVA) and the Student-Newman-Keuls test. A value of p<0.05 was considered to be statistically significant.

**RESULTS**

**Intrinsic antiviral activity**

The results presented in Figure 1 demonstrate that the intrinsic antiviral activity of monocytes obtained from patients undergoing HDF was significantly impaired compared with those obtained from the ND group. In particular, the extracellular virus release by monocytes from HDF postdialysis was higher than that from NDs (7.6×10^3 ± 908 vs. 7.7×10^2 ± 70 PFU/mL; p<0.05) as well as from HDF predialysis (7.6×10^3 ± 908 vs. 2.68×10^3 ± 319 PFU/mL; p<0.05). Furthermore, the stimulation of monocytes with 1 µg/mL LPS led to a significant decrease of virus replication only in monocytes from ND (7.7×10^2 ± 70 vs. 2.4×10^2 ± 32 PFU/mL; p<0.05).

**Cytokine production**

To assess whether the impaired antiviral activity observed in monocytes obtained from patients undergoing HDF could be related to an immunodysregulation mechanism, the production of different cytokines involved in the immune surveillance toward virus infection, such as IFN-α, IL-12 and TNF-α, was evaluated. The results concerning TNF-α (pg/mL) production are shown in Figure 2. No basal production of TNF-α was found in supernatants from ND monocytes, whereas appreciable amounts were detected in supernatants from pre-HDF and post-HDF monocytes (p<0.05). On the contrary, LPS stimulation increased 2-fold the production of TNF-α by monocytes from ND compared with that induced by monocytes from patients undergoing HDF (p<0.05). HSV-2 infection completely inhibited the TNF-α production by monocytes from patients undergoing HDF. On the other hand, when monocytes were stimulated with LPS and simultaneously...
infected with HSV-2, TNF-α production was down-regulated. The down-regulating effect of HSV-2 infection was statistically significant only on monocytes from pre-HDF and post-HDF patients (p<0.05) but not on monocytes from ND. Figure 3 shows that IFN-α was only detected in supernatants of monocytes stimulated with LPS, even though monocytes from ND produced higher amounts of IFN-α compared with monocytes from pre-HDF and post-HDF patients (138 ± 16 pg/mL for ND, vs. 21 ± 7 pg/mL and 16 ± 2 pg/mL, for pre-HDF and post-HDF, respectively; p < 0.05). When monocytes from ND were treated with LPS and simultaneously infected with HSV-2, IFN-α production was down-regulated (49 ± 9 pg/mL vs. 138 ± 16 pg/mL, p<0.05), whereas, in the same experimental conditions, monocytes from HDF patients did not produce detectable amounts of IFN-α.

As shown in Figure 4, no basal production of IL-12 was found in any of the supernatants assayed. Stimulation of monocytes with LPS induced the production of appreciable amounts of IL-12, even though monocytes from ND produced higher levels compared with monocytes from pre-HDF and post-HDF patients (239 ± 36 pg/mL vs. 82 ± 11 pg/mL and 36 ± 5 pg/mL, respectively; p<0.05). On the other hand, when monocytes were stimulated with LPS and simultaneously infected with HSV-2, IL-12 production was down-regulated. The down-regulating effect of HSV-2 infection was particularly evident on monocytes from post-HDF patients (36 ± 5 pg/mL vs. 8 ± 0.7 pg/mL). To verify whether these results were influenced by the incubation time and additional treatment, the viability of uninfected control cells was analyzed by MTT test. It was found that the different incubation times (24 and 44 hours) did not significantly influence the cell viability (data not shown).
DISCUSSION

It is well known that efficient elimination of viruses relies on the ability of the infected host to mount a proinflammatory immune response and develop a Th1-type immunity that in turn is able to restrict viral replication. This response is strictly characterized by activation of monocytes/macrophages, natural killer cells and cytotoxic T lymphocytes and production of proinflammatory cytokines and chemokines, including interferons, tumor necrosis factor-α and various interleukins such as IL-12 (16-19). Human monocytes show an intrinsic antiviral activity, defined as their ability to hinder intracellular viruses. Patients with end-stage renal failure undergoing hemodialysis show an impaired immune response with a high prevalence of viral complications. This dysregulated immune response may be related to shortened lymphocyte survival, lymphopenia, inhibition of lymphocyte differentiation and activity. However, the abundant literature on cytokines in hemodialysis is controversial. It has been shown that stimulation of cytokine gene transcription is not always followed by message translation and protein secretion. The matter is further complicated by high levels of circulating soluble receptors during dialysis (e.g. the TNF-α receptor) rendering the role of cytokines in hemodialysis-induced inflammation a complex and still debated issue (20-23).

Our results demonstrate that the intrinsic antiviral activity of monocytes obtained from patients undergoing HDF was significantly impaired with respect to that of monocytes obtained from healthy donors. To understand whether the depression of intrinsic antiviral activity could be related to an impairment of cytokine production, IFN-α, TNF-α and IL-12 were assayed in supernatants of monocytes in different conditions. Our results showed a significant reduction of cytokine production by monocytes from HDF patients compared with that observed in monocytes from healthy donors, even though a basal production of TNF-α was observed in monocytes from pre-HDF and post-HDF patients. Thus, the deficient antiviral activity could be related to a significant impairment of cytokine production. Our results seem to be not in agreement with other authors (24), who reported high levels of inflammatory cytokines in sera and blood samples drawn from ESRD patients (25, 26). However, in our opinion, this discrepancy could be due to our different experimental approach. In fact, the aim of this work was to analyze the in vitro intrinsic antiviral activity of purified monocytes obtained from PBMCs, as well as the release of cytokines usually involved in this mechanism of virus restriction, in patients undergoing HDF. We focused our studies on monocytes based on their pivotal role in proinflammatory response and their ability to directly impair viral replication as well. A number of other cell types including lymphocytes, that produce proinflammatory cytokines, were not included in our present in vitro study. The possibility that cytokines produced by other cell types could account for the high circulating levels found in ESRD patients cannot be ruled out. As a matter of fact, serum levels of cytokines in ESRD patients represent the product of many other cell types besides monocytes. Moreover, circulating cytokine levels may also be influenced by a decrease in their clearance, due to renal function impairment. In this preliminary study the actual level of circulating cytokines was not assessed in sera from patients. However, the eventual relationship between the “in vitro” production of cytokines by PBMCs of ESRD patients and their levels in sera is under investigation. The reduction in cytokine production in vitro by monocytes from patients appears to be related to a significant increase in viral replication. Moreover, HSV-2 infection completely inhibited the spontaneous release of TNF-α by monocytes from pre-HDF and post-HDF patients. Furthermore, HSV-2 infection of monocytes stimulated with LPS inhibited the production of all of the cytokines tested. These findings are in agreement with those reported by Mogensen et al (6), which demonstrated that HSV is able to suppress expression of proinflammatory cytokines by decreasing the stability of mRNAs, thereby potentially impeding the antiviral host response to infection. Even though this virus effect on the stability of mRNAs of monocytes from HDF patients cannot be ruled out, further studies are necessary to confirm the role of herpesvirus on stability of mRNA levels of proinflammatory cytokines in monocytes obtained from HDF patients. In fact, these mechanisms could be particularly detrimental in subjects with dysregulated immune response such as patients with end-stage renal failure undergoing HDF. Taken together, our data show that the significant increase in viral replication, observed in monocytes from HDF patients compared with that observed in monocytes from healthy donors, appears to be related to a significant reduction of cytokine production. Moreover, monocytes from HDF patients are hyporesponsive to LPS stimulation. In conclusion, HDF sessions may influence the intrinsic antiviral activity of monocytes, favoring replication of viruses and increasing the risk of opportunistic infections in patients with ESRD.
Conflict of interest statement: None declared.

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REFERENCES


