Abstract. The aim of this study was to evaluate the effect of both uremia itself and hemodialysis (HD) membranes on the induction of apoptosis. Four groups of subjects were evaluated: 21 nondialyzed (Non-D) patients, 10 continuous ambulatory peritoneal dialysis (CAPD) patients, and 53 HD patients who were on hemophan, cuprophan, cellulose acetate, AN69, and polysulfone; control subjects were nine healthy volunteers. Circulating mononuclear cells were obtained before dialysis and cultured for 48 h. Mean percentage of apoptosis was analyzed by a FACScan flow cytometer using Annexin V-FITC. Cell apoptosis was increased in Non-D patients (11.5 ± 5.5%) compared with control subjects (2.1 ± 0.7%, P < 0.001) and CAPD patients (7.0 ± 5.8%, P < 0.05). In patients on HD with cuprophan, apoptosis was higher than in control subjects and Non-D and CAPD patients. In Non-D patients, apoptosis was inversely correlated with renal creatinine clearance (r = -0.62, P = 0.003). Cell apoptosis was higher in hemophan than the other HD membranes. In seven patients on hemophan, switching to polysulfone resulted in decreased apoptosis (P < 0.01). Mononuclear cell circulation through mini-dialyzers made of different types of membranes (cuprophan, hemophan, cellulose acetate, AN69, and polysulfone) produced a significant increase in apoptosis. However, there was a marked difference in the percentage of apoptosis induced by these five membranes, being significantly increased in hemophan and cuprophan compared with the other three membranes. Similar results were obtained when whole blood from healthy donors was circulated through the mini-dialyzers, showing that mononuclear cell apoptosis was increased in hemophan and cuprophan compared with polysulfone. In conclusion, uremia and membrane characteristics may independently affect the mononuclear cell apoptosis.

Despite the technologic advances incorporated in the routine dialysis during the past few years, the mortality rate in the hemodialysis population remains elevated. Several reports have shown that compared with healthy individuals, the life expectancy of patients on replacement therapy for end-stage renal disease is markedly reduced (1,2). Several factors known to increase the risk of mortality in these patients are: poor nutrition, old age, high susceptibility to infections, long-time on dialysis and other comorbid conditions such as diabetes, hypertension, and cardiovascular disorders (3,4). Given the high rate of morbidity and mortality in dialysis patients, it is important to evaluate factors that might potentially improve the outcome of the dialysis population.

A beneficial effect of biocompatible membranes on hemodialysis patient survival has been recently reported (1,5,6). Studies based on a large number of patients show that the mortality rate of subjects dialyzed with unsubstituted cellulose membranes was higher than with synthetic and modified cellulose membranes (1). Interestingly, infection was one of the major causes of mortality associated with bioincompatible membranes (6,7). Impaired cellular host defense has been proposed to be one of the main mechanisms for increased susceptibility to infections in the dialysis population (8). However, the definite mechanism responsible for this host defense alteration is not well understood. Optimal host defense requires a fine balance between recruitment and death of immunocompetent cells; an alteration in the regulation of cell death by apoptosis may negatively affect the mechanism of host defense (9). We have shown that in vitro, the cell contact of bioincompatible membranes results in apoptosis (10).

Mononuclear cells play a major role in host defense and are one of the main factors responsible for the first step in the control of infection. Apoptosis of mononuclear cells may normally occur with aging or may be induced by inflammatory mediators such as cytokines (11,12). Hemodialysis therapy is challenged by the functional abnormalities derived from the contact of cells with membranes. During hemodialysis, mononuclear cells are stimulated resulting in interleukin-1 and TNF-α production and increased expression of adhesion molecules (13–16). Stimulation of mononuclear cells is likely caused by the interaction of cell-surface proteins with the dialysis membrane. In addition, stimuli related to the hemodialysis procedure may participate in cell activation (17). Hemodialysis-induced cell activation may result from cell contact with hemodialysis membrane plus other stimuli generated dur-
ing the hemodialysis procedure, such as complement factors derived from complement activation or the exposure to dialysate-borne bacterial product contamination (17,18). Thus, it seems that during hemodialysis with nonbiocompatible membranes, cell activation may be caused by more than one stimulus (10,14).

It is known that mononuclear cell apoptosis frequently occurs when cells are activated by two different stimuli or when they are subjected to an unphysiologic stimulation (10,19). These are conditions that may be present during hemodialysis when cells are exposed to membranes with a low degree of biocompatibility (10,20). In addition, recent reports have suggested that uremia may directly cause mononuclear cell apoptosis (12,19). The aim of the present study was to evaluate the influence of both uremia itself and different hemodialysis membranes on mononuclear cell apoptosis in vivo and in vitro.

Materials and Methods

Subjects

In a cross-sectional study, three different groups of end-stage renal failure patients were evaluated: uremic nondialyzed (Non-D) patients, continuous ambulatory peritoneal dialysis (CAPD) patients, and hemodialysis (HD) patients. The Non-D group included 21 subjects with advanced chronic renal failure before the initiation of chronic dialysis therapy. In this group of patients, the mean age was 50.8 ± 17.8 yr (range, 18 to 73), the mean renal creatinine clearance was 11.4 ± 4.2 ml/min, and all patients had a renal creatinine clearance <20 ml/min. The CAPD group consisted of 10 stable patients with a mean age of 59.5 ± 10.8 yr (31 to 69 yr) and an average time on dialysis of 40.4 ± 28.8 mo (15 to 107 mo). They had no episodes of peritonitis or catheter exit site infection during 6 mo before the study. The HD group included 53 patients with a mean age of 56.7 ± 11.2 yr (21 to 75 yr) and an average time on HD of 56.2 ± 44.3 mo (12 to 108 mo). The patients were dialyzed three times per week through arteriovenous fistulae; 47 were native and only six were polytetrafluoroethylene fistulae. The number of patients with polytetrafluoroethylene fistulae was similar in the five HD groups. These patients were evaluated before and 8 wk after being switched to a polysulfone membrane (HF80S; Fresenius). Informed consent was obtained from all patients after institutional approval.

Mononuclear Cell Preparation

Circulating human mononuclear cells were isolated from 10 ml of heparinized whole blood. In all patients, the blood samples were drawn immediately before the first HD session of the week (44 h since the last HD session), and patients were asked to fast the night before dialysis. In healthy volunteers, blood was obtained the morning after 8 h of fasting. Buffy coat cells were separated by differential centrifugation gradient (Ficoll/Hypaque; Pharmacia LKB, Uppsala, Sweden). Mononuclear cells were washed and seeded in 12-well culture plates with complete culture medium as described below. Monocytes were isolated from adherence to plates. A purity of >75% of cells was demonstrated by staining with anti-CD14 mononuclear antibody (mAb M5E2; PharMingen, San Diego, CA). Contamination with CD3+ and CD19+ (Leu-4 and Leu-12; Becton-Dickinson, Mountain View, CA) lymphocytes was <8%. Because monocytes were not absolutely pure after enrichment, representative control experiments were performed in cells isolated by flow cytometry and sorted using a monoclonal antibody against the CD14 molecule (AB383; R&D Systems, Abingdon, Oxon, United Kingdom). All maneuvers were done under strict sterile conditions.

Cell Culture

Cells were cultured in RPMI 1640 cell culture medium supplemented with l-glutamine (2 mM), HEPES (20 mM), sodium pyruvate (1 mM), streptomycin (50 mcg/ml), penicillin (100 U/ml), and 10% fetal calf serum (FCS) at 37°C in 5% CO2/95% air atmosphere. Fetal calf serum was heated during 1 h at 56°C to eliminate the complement-activating fractions. Cells were cultured in 96-well microtiter plates (Falcon; Becton Dickinson, Lincoln Park, NJ) at 2 × 10³ cells per well for 48 h.

Cell Apoptosis

Cell apoptosis was measured by Annexin V staining. One of the cell membrane changes during the early and intermediate stages of cell apoptosis is the translocation of phosphatidylserine from the inner side of the cell membrane to outside. Annexin binds only to cells in which phosphatidylserine has been translocated to the outside membrane. To evaluate apoptosis, cells were washed in phosphate-buffered saline and density was adjusted to 5 × 10⁵/ml. Then, cells were resuspended in binding buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂; filtered through a 0.2-μm filter); 5 μl Annexin V FITC (Bender MedSystems, Vienna, Austria) was added to 195 μl of cell suspension. After 10 min of incubation in the dark, cells were washed and resuspended in 190 μl of binding buffer and 10 μl of propidium iodide stock solution (20 μg/ml). The degree of apoptosis was assessed by flow cytometry. Live cells were considered those cells that were negative for both dyes, dead cells were positive for both fluorochromes, while apoptotic cells were positive only for Annexin V FITC and negative for propidium iodide. Background fluorescence was determined by FITC-conjugated mouse immunoglobulins.

In Vitro Study

To evaluate the independent effect of the dialysis membrane on the induction of apoptosis, and exclude other potential confounding fac-
tors, THP-1 cells and whole blood from healthy donors were circulated through a mini-dialyzer.

**Cell Culture**

THP-1 cells, a human mononuclear cell line (American Type Culture Collection, Manassas, VA), were cultured at 37°C in RPMI 1640 supplemented with l-glutamine (2 mM), Hepes (20 mM), sodium pyruvate (1 mM), streptomycin (50 μg/ml), penicillin (100 IU/ml), and 10% fetal bovine serum. Fetal bovine serum was preheated at 56°C for 60 min to inactivate complement.

**Reagents**

RPMI 1640, l-glutamine, hepes, Sodium pyruvate, streptomycin, penicillin, and fetal bovine serum were purchased from BioWhittaker (Walkersville, MD). Propidium iodide and DNase-free RNase A were purchased from Sigma Chemical Co. (St. Louis, MO). FITC-labeled Annexin V was purchased from Boehringer Mannheim (Mannheim, Germany). Proliferator-activated receptor (PARP)-specific monoclonal antibody and DNase-free RNase A were purchased from BioMOL (Plymouth Meeting, PA). 

**Statistical Analyses**

Results are expressed as mean ± SD. Nonparametric data were compared by Kruskal–Wallis test. Comparison between two means was analyzed by Mann–Whitney test for unpaired data and Wilcoxon signed rank test for paired data. Spearman rank correlation test was used for correlation analysis. Differences were considered significant at P < 0.05.

**Results**

**Effect of Treatment Modality on Cell Apoptosis**

Table 1 shows the main demographic characteristics of the subjects included in the study. The three groups of patients were not significantly different regarding age, gender, body weight, time on dialysis, and etiology of chronic renal failure.

The mean percentage of cell apoptosis was significantly elevated in Non-D patients (11.5 ± 5.5%) compared with control subjects (2.1 ± 0.7%, P < 0.001) and CAPD patients (7.0 ± 5.8%, P < 0.05). In patients with chronic renal failure on regular HD with cuprophan, the percentage of apoptosis (21.1 ± 12.6%) was significantly higher than in control subjects (P < 0.01) and Non-D (P < 0.01) and CAPD (P < 0.01) patients. Cell apoptosis in CAPD patients was higher than in control subjects, but differences did not reach statistical significance (Figure 1). In Non-D subjects, the renal creatinine

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Age (yr)</th>
<th>Gender</th>
<th>Time on Dialysis (mo)</th>
<th>Kt/V</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-D</td>
<td>50.8 ± 17.8</td>
<td>8/13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAPD</td>
<td>59.5 ± 10.9</td>
<td>4/6</td>
<td>40.4 ± 28.8</td>
<td></td>
</tr>
<tr>
<td>HD</td>
<td>56.7 ± 11.2</td>
<td>20/33</td>
<td>56.2 ± 44.3</td>
<td></td>
</tr>
<tr>
<td>CU</td>
<td>58.4 ± 14.3</td>
<td>4/6</td>
<td>69.3 ± 52.3</td>
<td>1.28 ± 0.10</td>
</tr>
<tr>
<td>HEM</td>
<td>54.0 ± 15.2</td>
<td>6/9</td>
<td>55.6 ± 35.2</td>
<td>1.27 ± 0.08</td>
</tr>
<tr>
<td>CA</td>
<td>54.7 ± 14.3</td>
<td>3/4</td>
<td>46.5 ± 41.3</td>
<td>1.30 ± 0.10</td>
</tr>
<tr>
<td>AN69</td>
<td>57.8 ± 13.3</td>
<td>4/8</td>
<td>56.6 ± 39.4</td>
<td>1.28 ± 0.09</td>
</tr>
<tr>
<td>PSF</td>
<td>59.1 ± 4.3</td>
<td>3/6</td>
<td>74.8 ± 47.9</td>
<td>1.26 ± 0.09</td>
</tr>
</tbody>
</table>

* Non-D, uremic nondialyzed; CAPD, continuous ambulatory peritoneal dialysis; HD, hemodialysis; CU, cuprophan; HEM, hemophan; CA, cellulose acetate; AN69, polyacrylonitrile AN69; PSF, polysulfone.

Figure 1. Effect of renal replacement therapy on mononuclear cell apoptosis. Results are expressed as mean percentage of cell apoptosis in healthy control subjects (C) and in patients receiving three different types of therapy: uremic non-dialyzed (Non-D), continuous ambulatory peritoneal dialysis (CAPD), and cuprophan hemodialysis (HDCU). *P < 0.01 versus controls; †P < 0.05 versus Non-D; ‡P < 0.05 versus CAPD.
clearance and blood sample for apoptosis were obtained on the same day. The results show that in these patients, apoptosis was inversely correlated with the renal creatinine clearance ($r = -0.62, P = 0.003$) (Figure 2).

**Effect of Dialysis Membrane on Apoptosis**

The effect of the HD membrane on mononuclear cell apoptosis was evaluated in uremic patients dialyzed with different types of dialyzers. These patients were not significantly different with regard to age, gender, etiology of chronic renal failure, body mass index, time on dialysis, duration of dialysis, dose of dialysis, and erythropoietin therapy.

Figure 3 shows the results obtained with the five types of dialyzers evaluated. The percentage of apoptosis was high in hemophan (29.3 ± 13.3%) and cuprophan dialyzers (21.1 ± 12.6%) and relatively low in AN69 (14.9 ± 9.3%) and polysulfone (15.6 ± 8.2%) membranes. The values observed with hemophan were significantly increased compared with controls ($P < 0.01$), Non-D ($P < 0.001$), CAPD ($P < 0.01$), and with the rest of the dialysis membranes ($P < 0.05$). The percentage of apoptosis with cuprophan membranes was greater than with the other three membranes, but the difference was not statistically significant. The percentages of cell apoptosis with cellulose acetate, polysulfone, and AN69 were significantly increased compared with controls ($P < 0.01$) and CAPD ($P < 0.05$), but similar to Non-D.

In seven patients chronically hemodialyzed with hemophan membranes, the change to polysulfone dialysis for an 8-wk period resulted in a marked decrease of apoptosis (from 25.7 ± 7.1 to 9.8 ± 5.0%, $P < 0.01$) (Figure 4).

**In Vitro Experiments**

To evaluate an independent effect of dialysis membrane on cell apoptosis, we performed additional *in vitro* experiments. The same five dialyzers tested *in vivo* were evaluated *in vitro*. Studies were performed using a human mononuclear cell line THP-1. Figure 5a shows the percentage of apoptosis before and after the circulation of the cells for 120 min through the five dialyzers. At baseline, the percentage of apoptosis was similar for all membranes. A significant increase of the apoptosis was observed in the five membranes after 120 min of cell circulation through the dialyzer: cuprophan (baseline 8.0 ± 1.8% versus 120 min 35.7 ± 3.3%, $P < 0.001$); hemophan (9.2 ± 3.5% versus 41.5 ± 3.0%, $P < 0.001$); cellulose acetate (8.2 ± 1.0% versus 16.5 ± 3.7%, $P < 0.01$); AN69 (6.3 ± 2.0% versus 14.3 ± 2.0%, $P < 0.01$), and polysulfone membranes.
membranes at 120 min. (B) The in vitro column represents the mean during a 2-h period. Left column shows the results of the control. Each blood from healthy donors was circulated through the mini-dialyzers membranes (CU, HF, and PSF) on mononuclear cell apoptosis. Whole age of apoptosis was similar in all membranes. A significant increase mean of apoptosis was observed in CU and HF compared to the other dialyzers (P<0.05). However, at 120 min, there was not a significant difference among the other three membranes tested.

Additional experiments in which whole blood from healthy donors was circulated through the mini-dialyzers for 2 h showed that the mononuclear cell apoptosis was significantly increased in hemophan (35.6 ± 6.5%) and cuprophan (30.5 ± 4.8%) versus polysulfone membrane (8.8 ± 1.0%) and control (5.6 ± 2.4%) (Figure 5b).

Discussion

Uremic patients have an increased incidence of infections and malignancies; both events are attributed to a defect in the immune system due to the uremic state and/or a direct consequence of the dialysis therapy (7,8,21,22). In the present study, a high degree of mononuclear cell apoptosis was observed in uremic patients compared with normal individuals. When the uremic patients were treated with dialysis, the percentage of apoptosis changed depending on the modality of dialysis used; apoptosis decreased with CAPD and increased with HD. Moreover, in HD patients cell apoptosis was influenced by the type of dialysis membrane, being greater with hemophan than with AN69 and polysulfone dialyzers. In addition, these results were confirmed by in vitro experiments, which reproduced the results observed in the HD patients.

During the HD procedure, the blood is exposed to the dialysis membrane resulting in mononuclear cell activation (10). Events associated with conventional HD, such as complement activation, upregulated expression of cell surface integrins, and release of proinflammatory cytokines, are related to cell membrane interaction (13,23–27). Uncontrolled monocyte activation may cause adverse effects; apoptosis may be a mechanism by which these chronically activated cells are deleted from the system. Previous studies by our group demonstrated that cell apoptosis might occur as the direct result of membrane-induced cell activation (10). The results obtained in the present study are in agreement with our recently reported in vitro data, which show that the interaction between mononuclear cell and cuprophan membranes resulted in cell aggregation and apoptosis. This effect was enhanced by preactivation of protein kinase C (10); and, in a different study, it was demonstrated that cuprophan-induced apoptosis was inhibited by pertussis toxin-susceptible G proteins (20). The results of these experiments suggest that the cell contact with cuprophan membrane can be transduced through cell surface proteins into specific intracellular apoptotic signals.

Independently of HD, the present study shows that uremia per se induces apoptosis. This is in agreement with a previous study performed by Heidenreich et al. (28). The normal immune response requires a fine balance between proliferation and cell deletion by apoptosis. We reason that both uremia and HD may affect the immune response by increasing the cell apoptosis.

There is no clear clinical evidence demonstrating a close relationship between the chronic activation of monocytes and the long-term complications of the HD patients (1,6,29–31). It has been proposed that some of the clinical adverse effects in long-term HD patients are due to the release of cytokines such as interleukin-1β, interleukin-6, and TNF-α during the HD procedure (14,17,25,32,33). However, these cytokines have been found to be elevated in uremic nondialyzed patients, indicating that monocyte stimulation may be induced by uremia itself, and therefore it is not related to the dialysis procedure only (34,35). The fact that there is a negative correlation between renal creatinine clearance and apoptosis suggest that uremia itself is in part the responsible for the increase in
apoptosis observed in the HD patients. It is likely that in these uremic patients, cell stimulation induced by the HD membrane was able to provoke a further increase in the apoptosis.

The second aim of this study was to evaluate the role of membranes on the induction of mononuclear cell apoptosis. The percentage of apoptosis was higher with hemophan than AN69 and polysulfone. In dialyzed patients with cuprophan, the percentage of apoptosis was greater than with AN69 and polysulfone, although this difference was not statistically significant ($P = 0.07$). It is important to note that the period of time required for observing a decrease in apoptosis after switching from hemophan to polysulfone was only 8 wk.

Heidenreich et al. (28) reported an acute decrease in the percentage of apoptosis in end-stage renal patients after the HD procedure independently of the membrane used. Therefore, it seems that the correction of uremia was able to reduce apoptosis. It is likely that the induction of apoptosis is related to two different stimuli: uremia and type of membrane. To separate both effects, additional experiments were designed using a human mononuclear cell line and healthy whole blood circulating through mini-modules with the same membranes that were used in vivo. Interestingly, the results from the in vitro experiments were quite similar to the in vivo observations: a high degree of apoptosis with hemophan and cuprophan, and relatively low apoptosis with polysulfone. These results were reproduced in vitro using both THP-1 cells and whole blood. The properties of polysulfone membranes made by various manufacturers may differ from each other; therefore, the results of the present study may not be extended to all polysulfone dialyzers.

In summary, our results show that both uremia and HD induce an increase in cell death by apoptosis. By contrast, CAPD treatment decreases apoptosis. The percentage of apoptosis observed in HD patients depends on the type of membrane used, being significantly increased with hemophan dialyzers. The present study supports the hypothesis that both the uremia itself and the properties of HD membrane have an effect on monocyte apoptosis. In conclusion, cell apoptosis seems to be closely related to the severity of uremia and type of dialysis therapy. A more complete understanding of the role that apoptosis plays in the pathophysiology of end-stage chronic renal failure may lead to new strategies for renal replacement therapy. Differences in the degree of apoptosis may help to explain why HD with a biocompatible membrane can reduce the morbidity and mortality of uremic patients with chronic infection episodes.

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