ization of LN status in CRC. An automated, multimarker QRT-PCR assay for characterization of LNs from CRC patients could be very useful for improved staging and treatment decision-making in CRC.

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References

Cell-Free Plasma DNA: A Marker for Apoptosis during Hemodialysis, Johanna Atamaniuk, Katharina Ruzicka, Karl M. Stuhlmeter, Alireza Karimi, Manfred Eigner, and Mathias M. Mueller (1) Institute of Laboratory Diagnostics, and (2) First Medical Department, Dialysis Unit, Kaiser Franz Josef Hospital, Vienna, Austria; (3) Ludwig Boltzmann Institute for Rheumatology, Vienna, Austria; (4) address correspondence to this author at: Institute of Laboratory Diagnostics, Kaiser Franz Josef Hospital, Kundratstrasse 3, A-1100, Vienna, Austria; fax 43-60191-3309, e-mail johanna.atamaniuk@wienkav.at

Background: We evaluated whether cell-free plasma DNA might be an appropriate marker for cell damage during hemodialysis (HD) and whether it correlated with annexin V expression and 7-aminomethylcoumarin D (7AAD) nuclear staining of blood leukocytes.

Methods: Circulating DNA, annexin V, and 7AAD were measured in HD patients before HD, 20 min after start of HD, and after HD had ended. Healthy volunteers provided control measurements. Necrosis and apoptosis were monitored by gel electrophoresis.

Results: Plasma DNA concentrations were not significantly different between controls and patients before HD. Circulating DNA increased significantly (P <0.05) after 20 min of treatment with HD. Post-HD concentrations of DNA were significantly higher compared with pre-HD and controls (P <0.005). Agarose gel electrophoresis showed ladders typical of apoptosis in post-HD samples. Two subpopulations of CD45+ leukocytes were defined by flow cytometry: annexin V+/7AAD+ population for apoptosis, and annexin V+/7AAD− for early apoptosis. Compared with healthy controls, mean fluorescence (MF) of 7AAD+ apoptotic cells in the annexin V+/7AAD+ population for apoptosis, and annexin V+/7AAD− for early apoptosis. Compared with healthy controls, mean fluorescence (MF) of 7AAD+ apoptotic cells in the annexin V+/7AAD+ population for apoptosis, and annexin V+/7AAD− for early apoptosis. Compared with healthy controls, mean fluorescence (MF) of 7AAD+ apoptotic cells in the annexin V+/7AAD+ population for apoptosis, and annexin V+/7AAD− for early apoptosis.

Conclusions: During HD, cell-free plasma DNA concentrations, annexin V expression, and 7AAD uptake in
leukocytes increases. The increase in plasma DNA, appearing as ladders typical of apoptosis, and the 7AAD uptake in leukocytes demonstrate that the predominant portion of circulating DNA in HD patients originates from apoptotic leukocytes.

Membranes used in hemodialysis (HD) are made of cellulose (e.g., cuprophan), modified cellulose (e.g., cellulose acetate), or synthetic polymers (e.g., polysulfone). Studies based on a large number of patients have shown that the mortality rate of individuals undergoing HD with unsubstituted cellulose membranes was higher than with synthetic or modified cellulose membranes (1). During HD, blood-membrane interactions lead to activation of circulating cells, plasma proteins, (2), and the complement system (3). Furthermore, contact of cells with less biocompatible membranes in vitro can lead to apoptosis (4). Exposure of normal neutrophils to uricemic plasma accelerates in vitro apoptosis compared with cells incubated with normal plasma (2). The apoptosis-inducing activity of uremic plasma is modulated by use of dialyzers with different degrees of biocompatibility (2). Dialysis membranes can promote neutrophil apoptosis directly as well as through their interactions with monocytes (5).

Stimulation of mononuclear cells is likely caused by the interaction of cell-surface proteins with the dialysis membrane (6).

For detecting early and late apoptosis in leukocytes, complex methods are required, such as flow cytometric measurements of annexin V expression in combination with 7-amino-actinomycin D (7AAD) nuclei staining (6).

In early apoptosis, phosphatidylserine (PS) is translocated from the inner to the outer surface of the plasma membrane. In the presence of Ca$^{2+}$, annexin V has a high affinity for PS and binds only to PS that has been exposed (7). Cells in the late stages of apoptosis and dead cells have lost plasma membrane integrity and are permeable for 7AAD (8). Increased apoptosis in leukocytes after HD was reported when they were cultured for 12 to 48 h (2, 6, 9).

Cell-free DNA concentrations are sensitive indicators of cellular damage originating from apoptosis or necrosis (10–12). The aim of this study, therefore, was to evaluate whether circulating DNA may be an appropriate marker to demonstrate apoptosis during HD and whether it correlates with annexin V expression and 7AAD nuclear staining of blood leukocytes.

Blood samples were drawn from a control group of 30 healthy donors (10 men and 20 women; age range, 25–75 years) and from 10 HD patients (5 men; age range, 61–74 years; 5 women; age range, 46–80 years) before HD, 20 min after the start of HD, and after the end of HD. All patients had been treated in the dialysis program for more than 2 weeks; the longest period of dialysis treatment was 42 months. In the HD group, 8 patients were treated 3 times and 2 patients 2 times per week. The length of time per HD procedure was 3.5–4.5 h. Informed consent was obtained from all patients.

For HD, the same synthetic polymer membranes [Fresenius Polysulfone Capillary dialyzers (F6 low-flux)] were used, and all patients were dialyzed through graft arteriovenous fistulas. The dialysate fluids and the water purity were routinely tested for bacteriologic contaminations and heavy metals. We administered the following medications to patients: parenteral iron [100 mg iron(III)-saccharose]; oral vitamins B$_1$, B$_2$, B$_6$, and C, folic acid, biotin, and nicotinamide; and subcutaneous erythropoietin (1000–15000 IU). Exclusion criteria for our study were status post-kidney transplantation, autoimmune disease, malignancy, or acute infection.

We isolated DNA from 800 μL of plasma and measured it as published previously (10). After isolation and measurement of plasma DNA, agarose gel electrophoresis was performed. Agarose, at a final concentration of 1.5% dissolved in Tris-borate-EDTA buffer (pH 8.0), was used for electrophoresis, and after electrophoresis, gels were stained with Vistra Green (Amersham Pharmacia Biotech). In cases where higher sensitivity was needed, polyanacrylamide gel electrophoresis was performed. Isolated plasma DNA samples were collected from 20 healthy volunteers and used after concentration (Microcon YM-100). The samples were separated on a 6% polyanacrylamide gel [30% acrylamide/bis (Bio-Rad/Laboratories); 5% buffer (50 mmol/L Tris, 380 mmol/L glyc-erin, 2 mmol/L EDTA)]; 100 μg/mL ammonium persulfate; 0.85 μL/mL N,N,N′,N′-tetramethylethylenediamine]. All chemicals were purchased from Sigma (Germany). Both gels were scanned on a FluorImager 595 (Amersham Biosciences).

To 0.5 mL of EDTA blood (4 °C), we added lysis buffer (8.99 g of ammonium chloride, 1.00 g of potassium hydrogen carbonate, 0.037 g of tritiplex-III, and 100.0 mL of doubly distilled water) diluted 1:10 with doubly distilled water. After incubation (5 min at room temperature in the dark) and centrifugation (300g for 5 min at 4 °C) to pellet the leukocytes, the cells were washed at 4 °C with phosphate-buffered saline (Dulbecco’s W/O sodium bicarbonate buffer; Gibco) supplemented with 10 mL/L fetal calf serum (PromoCell®), and then washed with ice-cold calcium buffer (10 mmol/L HEPES/NaOH, pH 7.4; 140 mmol/L NaCl; 2.5 mmol/L CaCl$_2$; IQ-Products) and adjusted to 1.5 × 10$^3$ cells/L.

We incubated 100 μL of cell suspension for 20 min in the dark at 4 °C with 10 μL of combined antibodies. Antibodies conjugated commercially to fluorescein isothiocyanate (FITC), phycoerythrin, peridinin-chlorophyll-protein (PerCP), and allophycocyanin (APC) dyes were used. The following tubes were provided: isotype control; anti-annexin V/FITC (IQ Products), 7AAD/PerCP (BD Pharmingen), and CD45/APC (Becton Dickinson); and anti-annexin V/FITC, CD45/phycoerythrin, 7AAD/PerCP, and CD14/APC. After incubation, the cells were washed and resuspended in ice-cold calcium buffer.

Surface marker analysis was performed by fluorescence-activated cell sorting with an FACSCalibur (Becton
Apoptosis, typically ladders appear in the DNA samples at the end of HD. (A), lane 1, molecular size ladder (100–1000 bp); lane 2, apoptosis control; lanes 3 and 4, plasma DNA samples immediately before dialysis and after 20 min of HD, respectively, showing only slightly increased ladders. Lane 5, for apoptosis, typically ladders appear in the DNA samples at the end of HD. (B), lane 1, molecular size ladder (100–1000 bp); lane 2, plasma DNA samples from 20 healthy volunteers concentrated into 1 sample. The pattern in lane 2 is typical of apoptotic ladders.

Dickinson). We acquired 10 000 events using CellQuest software (Becton Dickinson). We classified leukocytes as "normal" (annexin V− and 7AAD−), "early apoptotic" (annexin V+ and 7AAD−), and "apoptotic" (annexin V+ and 7AAD+).

Data were analyzed with STATISTICA for Windows, Ver. 6.0. Descriptive data are reported as the mean, median, and SD. Statistical significance was determined by Wilcoxon matched-pairs test for nonparametric variables. Statistical significance was defined as P < 0.05.

Before HD, plasma DNA concentrations in patients were slightly increased [mean (SD), 13.89 (5.22) pg/μL] compared with controls [12.53 (4.70) pg/μL]. After 20 min of HD, plasma DNA concentrations [20.45 (10.27) pg/μL] were significantly increased (P < 0.05) compared with pre-HD samples. During HD, plasma DNA continued to increase, with the highest concentrations at the end of HD [129.44 (83.38) pg/μL; P < 0.005].

Agarose gel electrophoresis of plasma DNA obtained from samples after HD revealed the typical apoptotic ladders. In pre-HD samples and in samples taken after 20 min of HD, we could detect only attenuated ladders (Fig. 1A).

To demonstrate whether the cell-free DNA of healthy individuals originates from apoptosis or necrosis, we performed polyacrylamide gel electrophoresis, using concentrated DNA extracts from 20 healthy individuals. These experiments again demonstrated typical apoptosis ladders (Fig. 1B). Thus, the predominant part of circulating DNA in healthy individuals originates from apoptosis.

To detect the mean fluorescence (MF) of annexin V and 7AAD, we monitored 2 distinct subpopulations of leukocytes: the annexin V+/7AAD+ (apoptotic), and annexin V+/7AAD− (early apoptotic) populations (Table 1).

The MF of 7AAD in the apoptotic cell population showed no significant differences between the controls and HD patients before start of HD. During HD, the MF of the 7AAD+ cells increased from 710.1 to 998.9 in patients’ samples. In addition, the MF of annexin V+ cells from patients before HD was significantly higher (P < 0.01) than in controls. During dialysis, we again detected significant changes in the MF of annexin V+ cells (P < 0.05) in patients. The early apoptotic cell subpopulations in controls and dialysis patients showed annexin V+ MF of 578.2 (131.5) and 662.2 (73.0), respectively (P > 0.05).

Uremia is associated with a state of immune dysfunction and increasing infection. Possibly, apoptosis relates to dysregulation of the immune system (13, 14). Cell-free plasma DNA has been found in many cases in which apoptosis or necrosis was involved, suggesting that such events are the main source for its presence. Measurement of circulating DNA has been used as a prognostic tool in the posttreatment monitoring of transplant patients (12). In addition, it has been shown that within 15 min to 3 h after major bodily injury, circulating DNA concentrations in the peripheral blood of trauma patients developing posttraumatic organ failure are significantly increased (15). In our previous study, we showed increased concentrations of plasma DNA immediately after exhaustive exercise and its disappearance within 2 h after the participants had stopped running (10). The rapid appearance

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![Fig. 1. Agarose gel separation of plasma DNA samples during HD (A), and polyacrylamide gel separation of concentrated cell-free plasma DNA samples (B).](image)

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Table 1. Flow cytometry results for CD45+ apoptotic leukocytes.

<table>
<thead>
<tr>
<th></th>
<th>7AAD+ (annexin V+/7AAD+ population)</th>
<th>Annexin V+ (annexin V+/7AAD− population)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>Controls (n = 20)</td>
<td>637.8</td>
<td>147.5</td>
</tr>
<tr>
<td>HD patients (n = 10)</td>
<td>710.1</td>
<td>144.9</td>
</tr>
<tr>
<td>Before HD</td>
<td>705.6</td>
<td>115.0</td>
</tr>
<tr>
<td>20 min of HD</td>
<td>998.9</td>
<td>428.9</td>
</tr>
</tbody>
</table>

* NS, not significant.

** Controls compared with before HD.

*a* Before HD compared with 20 min of HD.

*a,b* 20 min of HD compared with end of HD.
and disappearance of circulating DNA seems to be a characteristic phenomenon for the nature of this DNA.

Using DNA separated on a polyacrylamide gel, we have demonstrated that circulating DNA in healthy volunteers shows ladders typical for apoptosis. Circulating DNA seems to be mainly a result of apoptosis. In support of our hypothesis, Fournie et al. (16) concluded in a previous study that degradation of leukocytes in the artificial kidney was responsible for the increase in circulation of extracellular DNA.

Our data for 7AAD and annexin V in CD45+ leukocytes, measured immediately after blood draw, demonstrate apoptosis of leukocytes in HD. Previous in vitro studies (9) have demonstrated increased apoptosis of leukocytes after cultivation mimicking uremic conditions. In our study, increases in the MF of 7AAD in cell populations and in circulating DNA during HD mainly reflect apoptosis induced by contact with the dialysis membrane.

The source of increased DNA concentrations seems to be apoptotic leukocytes. Possibly, DNA fragments are leaking from the nuclei of leukocytes. In our study, the increase in uptake of 7AAD in leukocytes indicates increased permeability. The rapid increase in circulating DNA during HD and the increase in 7AAD in leukocytes suggest that both are caused by apoptosis.

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Background: In many countries, regulatory authorities that use International Organization for Standardization Standards to assess laboratory competence require an estimate of the uncertainty of measurement (MU) of assay test results. This estimate can be determined by identifying all sources of variation, calculating the extent of variation, and using established methods to combine the uncertainty. Alternatively, laboratory staff may use existing data generated from evaluations, proficiency testing, or external run controls to determine MU.

Methods: A quality-control (QC) sample with low reactivity was tested by laboratories participating in a national QC program. The results of testing the QC sample were entered into a shared database by use of an Internet-based program, EDCNet. Using a statistical approach that accounts for imprecision and bias of test results, we estimated the MU of the laboratories.

Results: A total of 2167 test results of a single QC sample reported by 18 laboratories were analyzed, and the MU of 1 laboratory was estimated by the statistical model described.

Conclusion: Using peer-group run control data, MU of serologic testing can be estimated by taking into account both imprecision and bias.

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Most regulatory authorities that use International Organization for Standardization (ISO) Standards to assess