Polymeric protein-polyamine conjugates: A new class of uremic toxins affecting erythropoiesis

FRANCESCO GALLI, SIMONE BENINATI, SERENA BENEDETTI, ALESSANDRO LENTINI, FRANCO CANESTRARI, ANTONIO TABILIO, and UMBERTO BUONCRISTIANI

"G. Fornaini" Institute of Biological Chemistry, University of Urbino, Urbino; Department of Biology, University of Rome "Tor Vergata," Rome; Department of Clinical and Experimental Medicine, Hematology Section, University of Perugia, and Nephrology and Dialysis Unit, "R. Silvestrini" Hospital, Perugia, Italy


Background. Preliminary evidence on the accumulation of polyamine-protein conjugates (PPCs) was obtained in uremic patients. The presence of these substances in the plasma of hemodialysis (HD) patients was evaluated, and their possible contribution to uremic anemia was investigated by testing the effect of PPC synthesized in vitro on erythroid cell proliferation.

Methods. Plasma PPC was measured by high-performance liquid chromatography. The in vitro synthesis of PPC from human plasma was carried out by means of the enzyme transglutaminase in the presence of either [³H]-labeled or unlabeled spermidine (SPD). After gel filtration chromatography and detection of the fractions containing [³H]SPD, the latter were tested for their effect on mononuclear bone marrow cell proliferation.

Results. In three out of four patients examined, mainly SPD-protein conjugates (SPD-PC) were observed to accumulate during HD. The levels ranged from 0.17 to 4.93 pmol/mg proteins before dialysis, and these values increased at 30 minutes and at the end of the dialysis up to levels 11.90 pmol/mg. SPD-PC levels in healthy controls were 1.46 ± 0.82. SPD-PCs synthesized in vitro were recovered in two main fractions showing a molecular weight of >100 kDa (peak 1) and of approximately 30 to 50 kDa (peak 3), respectively. The SPD-PC contained in peak 1 showed the greatest inhibitory effect on colony-forming units-erythroid (CFU-E) proliferation without any appreciable effect on burst-forming units-erythroid (BFU-E).

Conclusion. We demonstrate that SPD-PC can accumulate in HD patients. These substances, which affect CFU-E proliferation, can be considered as an at yet unrevealed class of uremic toxins contributing to the onset of the uremic anemia.

Uremic anemia is a severe, multifactorial state mainly dependent on erythropoietin (EPO) deficiency or unresponsiveness [1, 2]. Clinical evidence obtained to date has indicated that intense hemodialysis (HD), together with the use of highly permeable and biocompatible dialyzers, may increase the response to recombinant human EPO (rHuEPO) supplementation [2], the therapy of choice for this hypoproliferative anemia. Toxins accumulating in uremic serum may be key contributors to this disease state, possibly by interfering with the EPO receptor [3] or by directly damaging erythroid cells [4].

Higher than normal concentrations of extracellular polyamines (PAs) are known to exert a consistent hypoproliferative function on human erythroid precursors and particularly on the colony-forming units-erythroid (CFU-E) [5]. At the same time, some studies have suggested that plasma PA can accumulate in the uremic plasma and that their levels may be inversely correlated with the hematocrit values [5]. This evidence supports the hypothesis that PA can contribute to the genesis of uremic anemia.

Nearly 20 years ago, Lutz observed the presence of a spermidine (SPD) peptide in the dialysate and plasma from continuous ambulatory peritoneal dialysis (CAPD) patients [6]; however, this evidence was never carried further. Recently, the characterization of compounds eliminated by protein-leaking dialyzers led to our preliminary observation that polyaminated proteins can also accumulate in the plasma of some HD patients [7].

Despite these findings, the biochemical and toxicological features of these compounds are not yet understood. In the present study, we evaluated the presence of plasma PA-protein conjugates (PPCs) in end-stage renal disease (ESRD) patients on HD. Moreover, since PPC might share some of the toxicological features of free PA, we synthesized PPCs in vitro from human plasma to investigate their effect on the proliferation of bone marrow erythroid progenitors.

METHODS
Patient selection and blood sampling
After informed consent, four ESRD patients on HD stabilized in conditions of good metabolic equilibrium

1 Both authors contributed equally to this study.

Key words: anemia, CFU-E, hemodialysis, spermidine, erythroid cells, end-stage renal disease.
© 2001 by the International Society of Nephrology

S-73
were included in this study. Inclusion criteria were the absence of conditions interfering with the investigation, such as bone marrow failure caused by hyperparathyroid fibrosis or chronic or acute illness, severe infectious and inflammatory diseases, or hereditary enzymatic deficiencies. Any pharmacological treatment directly interfering with the red blood cell metabolism or erythropoiesis, with the exception of rHuEPO administration, was interrupted for a sufficient period of time before the beginning of the protocol.

At the start of the HD session, after 30 minutes and at the end of HD, 10 mL of blood were drawn from the arteriovenous fistula and collected in heparinized tubes. Plasma samples were separated by centrifugation and stored at -20°C until assessed.

Analysis and in vitro synthesis by transglutaminase of plasma PPCs

The conditions for the preparation of samples and the analysis of PPC in the plasma by reversed-phase high-performance liquid chromatography (RP-HPLC) were those utilized by Beninati, Martinet, and Folk [8].

Aliquots of plasma (120 µL) from both healthy controls and uremic patients were incubated for two hours at room temperature, according to a published procedure [9], in the presence of 80 µg/mL of purified guinea pig liver transglutaminase (TGase; Sigma Chemicals, Milano, Italy) and 5 µL (15 Ci/mmol) of SPD trihydrochloride, [terminal methylenes-3H(N)] (NEN, Boston, MA, USA) or 0.4 µg/mL of unlabeled SPD trihydrochloride (Sigma Chemicals). The same amount of plasma, incubated in the absence of TGase and [3H]SPD or unlabeled SPD, was utilized as control.

After the incubation, 20 mg of plasma proteins were directly loaded on a Sephadex G 200 column (Pharmacia Biotech, Cologno Monzese, Italy) and eluted with 0.1 mol/L phosphate buffer, pH 7.4, at a flow rate of 0.5 mL/min. Fractions of 2.5 mL were collected, and their absorbance at 280 nm was checked. In the case of the samples containing [3H]SPD, the radioactivity of the chromatographic fractions was detected by scintillation counting. Finally, fractions in which the incorporation of [3H]SPD into proteins was found to be significant were pooled and tested on bone marrow cells.

Bone marrow erythroid cell proliferation assay

Bone marrow samples were obtained from normal human volunteers after informed consent. Under sterile conditions, the disaggregated cell suspension was layered over Ficoll-Paque (Seromed, Berlin, Germany) and centrifuged. The washed pellet was resuspended in alpha medium (RPMI 1640; Sigma), and mononuclear cells were cultured at 37°C in an atmosphere of 5% CO2 at a final concentration of 20,000 cells/mL in a culture mixture (Methocult GF H4434; Stem Cell Tecnologies Inc., Vancouver, Canada) containing 0.9% methylcellulose in Iscove's modified Dulbecco's medium, completed with the stimulatory factors necessary for the growth of erythroid progenitors as provided by the manufacturer [10].

In the proliferation experiments, colony-forming units-erythroid (CFU-E) and burst-forming units-erythroid (BFU-E) colonies were evaluated using a light-inverted microscope after 7 and 14 days of culture, respectively. The effect of fractions obtained as described previously in this article (by gel filtration chromatography of plasma samples before and after reaction with TGase and SPD) was tested by diluting the same fractions in the culture medium (10% vol/vol).

RESULTS

Plasma levels of PPCs

High levels of PPCs were found in the plasma of uremic patients undergoing HD. In particular, SPD-protein conjugates (SPD-PC; Table 1) were observed to accumulate in three out of four patients examined, and during HD, they reached levels nearly 10 times those of healthy controls (1.46 ± 0.82 pmol/mg proteins, range 0.15 to 2.78). Before HD, the levels of SPD conjugates ranged from 0.17 to 4.93 pmol/mg proteins, but these levels were found to be higher at 30 minutes and at the end of the dialysis (between 0.17 and 11.90 pmol/mg proteins). The accumulation of these polyaminated proteins was observed regardless of the type of dialyzer used, although after dialysis the high-flux membranes (polysulphone and polymethylmethacrylate) seemed to have allowed the maintenance of lower levels as compared with those afforded by the cuprammonium rayon-based low-flux dialyzer.

In vitro synthesis of PPC

The chromatographic profile obtained after gel filtration of human plasma from both healthy controls and uremic patients showed three main peaks (Fig. 1A) corresponding (from left to right) to the IgG fraction (peak 1, M, 150 kD), albumin (peak 2, M, 66 kD) and a group of unidentified proteins here defined as "prealbumin fraction" (peak 3, M, 30 to 50 kD). After incubation with the enzyme TGase in the presence of [3H]SPD, the assay of the radioactivity in the chromatographic fractions revealed that SPD-PC accumulate in peaks 1 and 3, respectively.

The SDS-PAGE analysis (data not shown) of the fractions representative of the three main peaks mentioned previously in this article showed that peak 1 in the TGase-treated samples contained high molecular weight solutes not entering the separating gel (estimated molecular weight >200 kD), which were present at trace levels in the untreated samples. At the same time, at least three
Table 1. Levels of plasma spermidine-protein conjugates (SPD-PC; pmol/mg proteins) in four uremic patients on chronic hemodialysis (HD)

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Filter type</th>
<th>Hct %</th>
<th>rHuPO IU/week</th>
<th>Age years</th>
<th>Before HD</th>
<th>30 min HD</th>
<th>After HD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Polysulphone</td>
<td>30.7</td>
<td>4000</td>
<td>78</td>
<td>4.93</td>
<td>11.39</td>
<td>3.57</td>
</tr>
<tr>
<td>2</td>
<td>Cuprammonium rayon</td>
<td>37.9</td>
<td>—</td>
<td>66</td>
<td>3.06</td>
<td>5.44</td>
<td>11.90</td>
</tr>
<tr>
<td>3</td>
<td>Polymethylmethacrylate</td>
<td>32.4</td>
<td>2000</td>
<td>50</td>
<td>0.17</td>
<td>3.06</td>
<td>1.70</td>
</tr>
<tr>
<td>4</td>
<td>Cuprammonium rayon</td>
<td>31.6</td>
<td>12000</td>
<td>74</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

Age- and sex-matched healthy controls (N = 4) showed plasma levels of SPD-PC of 1.46 ± 0.82 pmol/mg proteins (range from 0.15 to 2.78). Abbreviations are: Hct, hematocrit; rHuEPO, recombinant human erythropoietin in International Units (IU) administered per week.

*SPD-PC were determined as SPD obtained from the acidic hydrolysis of the γ-glutamyl derivatives of plasma proteins [8]

---

** Fig. 1. Chromatographic analysis (A) and biological activity (B) of plasma proteins before and after incubation with transglutaminase (TGase) and [3H]-labeled spermidine ([3H]-SPD). The experiment shown, representative of the entire series of chromatographic analyses performed, was carried out as described in the text. (B) The erythroid cell proliferation assay performed in the presence of protein fractions collected from the gel filtration chromatography carried out as (A). Only proliferation data regarding CFU-E were shown (*t-test: P < 0.01).**

---

**different electrophoretic bands with Mᵋ ranging from approximately 32 to 55 kD were observed in peak 1 obtained from untreated plasma samples, but were undetectable in the corresponding peak of the TGase-treated plasma. Minor differences were found in the plasma protein pattern between controls and uremic patients both before and after the reaction with TGase (data not shown).**

**Hypoproliferative activity of the SPD-PC on bone marrow erythroid precursors**

A significant inhibitory effect on erythroid cell proliferation (Fig. 1B) was observed only when the fraction pool corresponding to peak 1 (Fig. 1A) from plasma samples treated with TGase was incubated with mononuclear bone marrow cells. This effect was highly specific for CFU-E (60% inhibition), with the BFU-E proliferation rate unaffected (data not shown). The same fractions from native plasma did not show any inhibitory effect on CFU-E growth.

**DISCUSSION**

In 1980, Lutz first observed the presence of a SPD peptide in the dialysate obtained from continuous ambulatory peritoneal dialysis patients [6], but this observation was never carried further until recently, when we made the preliminary observation that polyaminated peptides and proteins can accumulate in the plasma of HD patients [7]. This accumulation could contribute to the toxicity related to high molecular weight toxins, which are not eliminated with standard low-flux dialyzers [7, 11, 12]. Some of these high molecular weight substances, which are still largely unknown, have a specific inhibitory effect on CFU-E proliferation, and their removal by protein-leaking dialyzers can improve the anemic status of some HD patients [7, 13].

In the present study, we have confirmed the accumulation of PA in the uremic plasma of HD patients, demonstrating that SPD is the main species involved in the formation of these conjugates. SPD-PCs seem to be gen-
erated during dialysis, and in accordance with our previous preliminary observations [7], they appear to be eliminated, at least in a part, by high-flux dialyzers. Therefore, we can speculate that PPC could be one of the high molecular weight toxins contributing to the anemia of HD patients [7, 12, 13].

From a biochemical standpoint, the covalent bond between PA and plasma proteins is not a spontaneous event, but is specifically catalyzed by the enzyme transglutaminase (TGase), which in its active form leads to the incorporation of amines, including PA, into proteins in the form of amides of the γ-carboxyl group of a glutamic acid residue [14]. We hypothesize that two main classes of TGase might take part in the formation of PPCs observed in HD patients: the extracellular form present in the plasma and also known as blood coagulation factor XIII; and the intracellular forms (tissue TGase and particulate TGase). The formation of PPC might thus result from the activation of factor XIII or from the release of intracellular enzymes as a consequence of blood cell activation and/or lysis. Both events can be triggered during the HD session as a consequence of the contact of blood with the extracorporeal circulation and of changes in solute and pH levels. However, the possibility that other forms of the TGase enzyme different from those now known could lead to the formation of SPD-PC in uremia cannot be ruled out [6]. Further investigations on the identification of the source of the TGase and on HD factors leading to its activation are in progress.

However, the main aim of this study was to characterize the possible toxicological properties of PPC in uremia and in particular their effect on erythroid precursors. In fact, it is known that PA, in their free form, can be powerful inhibitors of the erythroid cell proliferation both in vitro and in vivo, leaving space for the hypothesis that PAs may be key contributors to the genesis of the uremic anemia [3].

This study provides the first evidence, to our knowledge, of a significant antiproliferative effect of the SPD-PC synthesized from plasma on erythroid progenitor CFU-E, which is independent from the presence of free SPD. This finding suggests that plasma SPD-PC found in HD patients might be a class of toxins participating to the onset and/or progression of their anemic status. Evidence that PPCs have no influence on BFU-E growth indicates that similar to free SPD, they mainly inhibit erythropoiesis by interfering with the EPO function (for example, by modifying specific EPO receptors). In fact, only CFU-Es, but not BFU-Es, respond to EPO due to specific receptors on the cell membrane.

Spermidine-PCs have also been proposed to interact with insulin and lipoproteins, possibly contributing to other factors of comorbidity of chronic uremia such as hypertriglyceridemia and accelerated atherosclerosis [6].

In conclusion, we demonstrate that SPD-PCs, which accumulate in at least a part of HD patients, affect CFU-E proliferation, thus playing a possible role in the genesis of the uremic anemia.

Reprint requests to Francesco Galli, Ph.D., Istituto di Chimica Biologica "G. Fornaini," Via Saffi 2, 61029 Urbino (PS), Italy. E-mail: galli@uniurb.it

REFERENCES


