Release of Granulocyte Proteinases during Hemodialysis

Walter H. Hörl[a], *Marianne Jochum*[b], *August Heidland*[a], *Hans Fritz*[b]

[a] Department of Medicine, Division of Nephrology, University of Würzburg, FRG, and
[b] Surgical Clinic, Department of Clinical Chemistry and Biochemistry, University of Munich, FRG

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Abstract. Neutral proteinases of neutrophilic polymorphonuclear leukocytes were followed up cytochemically in blood smears of 12 patients submitted to regular hemodialysis treatment (RDT). Halo formation (ring-shaped area around each neutrophil due to protein degradation) was reduced in all patients with end-stage renal disease under RDT. Concomitant to the development of leukopenia, a maximal increase of the plasma levels of the granulocytic elastase in complex with α_1-proteinase inhibitor was observed 3 h after starting hemodialysis (+409%; $p < 0.001$). On the other hand, the proteolytic activity of the plasma samples against azocasein as substrate, being significantly higher (+244%; $p < 0.001$) in RDT patients compared with healthy controls, decreased permanently during therapy (−71%; $p < 0.001$; 3 h after initiation of the treatment). The mechanisms of release as well as of elimination of proteolytic activity due to RDT are discussed.
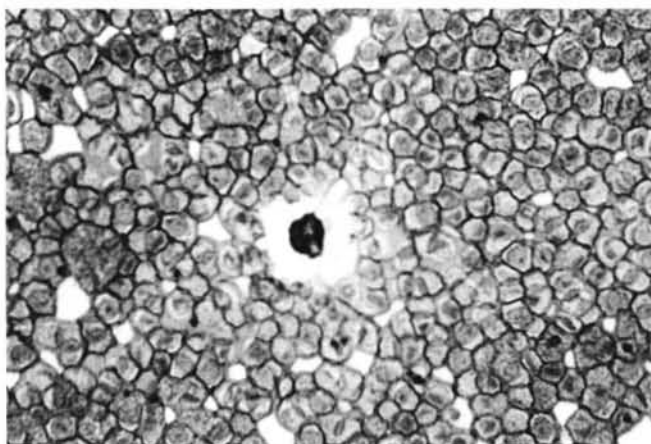
Introduction

Hemodialysis therapy is associated with a considerable loss of amino acids and glucose [1, 2]. On the other hand, hepatic gluconeogenesis is enhanced during renal insufficiency [3–5]. Two possible causes of the reported increase in intradialytic protein catabolism [6] would be gluconeogenesis to replace glucose loss (during glucose-free dialysis) and protein breakdown to replace amino acid loss [7]. Glucose was found to be ineffective in reducing dialysis-induced catabolism. The average intradialytic urea generation was increased by 28% with glucose-free dialysis compared with 24 and 25%, respectively, in the presence of glucose in the dialysate at a level of 11 or 26 mmol/l [7]. Furthermore, the replacement of the loss of amino acids during dialysis by continuous amino acid infusion did not lower intradialytic protein catabolism, but appeared to increase it [6].

Several studies have shown that proteinases participate in the protein catabolism of patients with hypercatabolic acute renal failure [8–11]. Therefore, we evaluated the possibility of enhanced protein breakdown by proteolytic enzymes liberated or activated during hemodialysis therapy. The rational for this is given by the high conc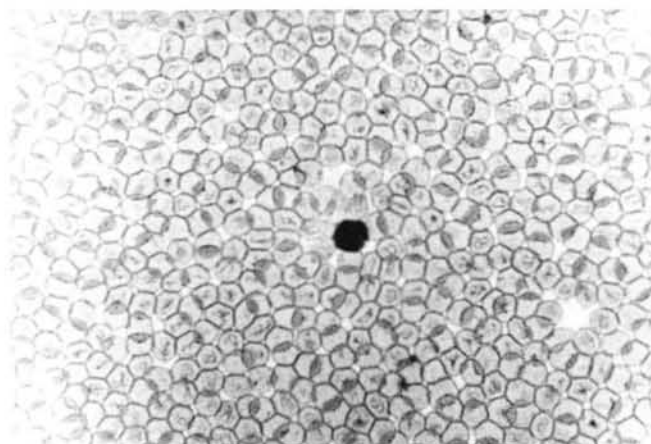entration of proteinases in neutrophil granulocytes and the well-known decrease of white blood cells followed by increased enzyme liberation after initiation of hemodialysis therapy – for review, see *Heidland* et al. [12]. The aim of our present study was twofold: (1) the cytochemical evaluation of neutral proteinases of neutrophilic polymorphonuclear (PMN) leukocytes of patients subjected to regular hemodialysis treatment (RDT), and (2) the detection of an increased liberation of the granulocytic proteinase elastase by measurement of the elastase-α_1-proteinase inhibitor (E-α_1PI) complex in plasma samples of RDT patients. The plasma level of this complex was shown to be a suitable indicator of elastase liberation from activated or desintegrated granulocytes.

Material and Methods

We report here the results obtained in 12 chronically uremic patients (9 men), aged 47.3 ± 4.0 years (mean ± SEM, range 22–66), undergoing RDT for 41.3 ± 11.6 months (range 4–124). Hemodialysis was performed three times per week for 5 h with Gambro Lundia Optima and a glucose-free bath containing acetate. 4 patients suffered from diabetic glomerulosclerosis, 2 from polycystic kidney degeneration and 6 from chronic glomerulonephritis. Blood cells were counted by an electronic counter (Coulter-Counter-Model B). Further blood constituents were determined as follows: creatinine 11.5 ± 0.8 mg/dl (range 7.2–16.9); urea nitrogen 76.9 ± 4.7 mg/dl (range 54–105); hemoglobin 9.2 ± 0.5 g/dl (range 7.4–13.2), and

Fig. 1. Blood smear from a healthy subject. Fixation was performed using formaline sublimate. The smear was incubated with 0.25 M NaCl borate buffer (pH 8.5) for 120 min at 37 °C and stained with colloidal iron.

Fig. 2. Absence of halo formation in a blood smear of a RDT patient 30 min after initiation of hemodialysis therapy. For incubation conditions see legend to figure 1.

hematocrit 30.3 ± 1.7% (range 23.7–42.5). Plasma samples were obtained by withdrawing 4.5 ml of venous blood into plastic syringes containing 0.5 ml of sodium citrate (2.2 g/100 ml distilled water). Plasma was separated from blood within 30 min after sample collection to prevent leakage of leukocyte constituents. The plasma specimens were stored at −70 °C until assayed.

The inhibitory activity of antithrombin III (AT III) against thrombin was determined using the thrombin-specific chromogenic substrate S-2238 (Deutsche Kabi, Munich, FRG). The inhibitory activity of the α_1-proteinase inhibitor (α_1PI = α_1-antitrypsin) was measured with a commercial test system (Boehringer, Mannheim, FRG). Plasma concentrations of α_2-macroglobulin (α_2M), α_1PI, albumin as well as complement components C_3 and C_4 were evaluated by a radial immunodiffusion technique with standardized immunodiffusion plates (Behringwerke AG, Marburg, FRG).

Quantitative estimation of the plasma levels of the E-α_1PI complex was carried out with a highly sensitive enzyme-linked immunoassay [13]. Proteolytic activity of the plasma samples was measured using azocasein as substrate as previously described [9, 14]. Proteinase activity of the neutrophilic PMN leukocytes was observed with the cytochemical test of *Klessen* [15].

Results were expressed as mean values ± SEM. Statistical evaluation was performed by the Student's t test.

Results

The cytochemical test of *Klessen* [15] was used to evaluate the activity of neutrophilic PMN leukocyte proteases. These enzymes are released from the PMN neutrophils of healthy subjects in blood smears following incubation with sodium chloride borate buffer. Proteolytic activity is revealed by a ring-shaped area around each neutrophil (fig. 1). This halo effect is due to erythro-

cyte hemolysis and plasma protein degradation. Halo formation was reduced in the blood smears of all patients with end-stage renal disease undergoing RDT (fig. 2).

The effect of hemodialysis on leukocytes, plasma levels of the E-α_1PI complex as well as on α_1PI activity and concentration is shown in table I. There was a decrease of white blood cells 10 min (21.1%; n.s.) and 30 min (41.1%; p < 0.001) after initiation of hemodialysis therapy. We observed maximum levels of the plasma E-α_1PI complex after 3 h (+409%; p < 0.001). However, plasma α_1PI activity and concentration were unchanged during hemodialysis therapy.

The plasma levels of α_2M (before dialysis, 244 ± 17; after 3 h of dialysis therapy, 257 ± 31 mg/dl), AT III (91.3 ± 2.5 vs. 95.2 ± 4.3%), complement components C_3 (154 ± 6 vs. 160 ± 8 mg/dl) or C_4 (61.7 ± 3.8 vs. 64.6 ± 3.7 mg/dl) remained also unchanged. Dialysis therapy was performed to maintain a constant body weight during the first 3 h. Plasma albumin concentration (3.77 ± 1.12 vs. 3.88 ± 0.14 g/dl) or hematocrit (30.3 ± 1.7 vs. 31.3 ± 2.0%) were in the same range before and 3 h after starting hemodialysis therapy.

Using azocasein as a substrate, the proteolytic activity of the plasma samples was significantly higher (+244%; p < 0.01) before dialysis therapy compared with plasma samples of healthy controls (0.052 ± 0.004 U/mg protein). During dialysis therapy, however, there was a permanent decrease of this plasma proteinase activity (0.127 ± 0.019 vs. 0.037 ± 0.007 U/mg; −71%; p < 0.001; table I).

Table I. Effect of hemodialysis therapy on leukocyte counts, plasma E-α_1PI complex, proteinase activity as well as on activity and plasma concentration of α_1PI

	Time, min					
	0	10	30	60	120	180
Leukocytes, cells/mm³	$7{,}133 \pm 582$	$5{,}625 \pm 696$	$4{,}200 \pm 251^c$	$6{,}658 \pm 441$	$5{,}950 \pm 607$	$7{,}192 \pm 824$
E-α_1PI, ng/ml	188 ± 20	196 ± 25	326 ± 56^a	373 ± 32^c	602 ± 84^c	769 ± 128^c
Proteinase activity, U/mg protein	0.127 ± 0.019	0.103 ± 0.014	0.095 ± 0.014	0.087 ± 0.013	0.059 ± 0.010^b	0.037 ± 0.007^c
α_1PI, U/ml	1.98 ± 0.11	1.89 ± 0.11	2.07 ± 0.10	2.06 ± 0.09	2.01 ± 0.09	2.08 ± 0.11
α_1PI, mg/dl	254 ± 14	257 ± 13	268 ± 13	258 ± 12	260 ± 11	271 ± 13

Mean values $\pm$ SEM from 12 experiments before and during hemodialysis therapy.
[a] $p < 0.05$; [b] $p < 0.01$; [c] $p < 0.001$.

Discussion

It has been shown recently that glucose in the dialysate [7] as well as continuous amino acid infusion [6] are ineffective in preventing the pathological catabolic events observed during hemodialysis. The pathogenesis of hemodialysis-induced protein catabolism remains so far unclear. One pathogenetic factor could be the release of granulocyte proteinases after starting hemodialysis therapy. *Craddock* et al. [16] have demonstrated hemodialysis-induced leukopenia and pulmonary vascular leukostasis resulting from complement activation by dialyzer cellophane membranes.

Neutrophil granulocytes contain a broad variety of agents that are involved in the defence and digestion of invading microorganisms [17]. These include elastase [17, 18], cathepsin G, cathepsin B, cathepsin D and collagenase [19–24]. Lysosomal proteinases are not exclusively restricted to their intracellular compartment, the lysosomes. They may be released intra- as well as extracellularly during phagocytosis, exposure to antigen-antibody complexes, complement components and toxic substances such as endotoxins or during cell death [25, 26]. Especially under pathological conditions massive release of proteases and other lysosomal factors may occur. They might cause tissue injury [27, 28] and consumption of plasma proteins by unspecific proteolytic degradation as soon as the levels of the controlling proteinase inhibitors in plasma and tissues are insufficient [29].

Granulocyte elastase is the most potent candidate for unspecific degradation because of its broad substrate specificity and the large amount present in the PMN granulocytes – for review, see *Heidland* et al. [12]. It degrades various plasma proteins such as transferrin [*Jo-chum and Lander,* unpublished data], immunoglobulins [30, 31] and fibronectin [32]. In vitro elastase rapidly inactivates fibrinogen [33, 34] and several other clotting factors [35, 36]. It also causes a limited degradation of the third and fifth factor of human complement [37, 38].

Granulocyte elastase could be released during hemodialysis due to the contact of blood cells with the blood lines and the dialyzer membrane (cuprophane). Such contact may result in a so-called 'frustrated phagocytosis' known to be associated with an extracellular release of lysosomal proteinases. The reduced proteolytic activity of PMN leukocytes in blood smears obtained 30 min after onset of hemodialysis and the concomitant increase of E-α_1PI plasma levels may be regarded as possible indicators of this 'escape' reaction. On the other hand, proteinase release due to cell death must be also taken into account. Since, however, leukopenia does not strictly parallel the increase of the E-α_1PI levels during hemodialysis, pulmonary vascular leukostasis resulting from complement activation by dialyzer cellophane membranes may be an additional source of local lysosomal enzyme release [16]. This event could indeed be responsible for the delayed release of elastase into the circulation. Although plasma activity and concentration of α_1PI, the dominating elastase inhibitor [39], were unchanged during hemodialysis therapy (table I), the liberation of granulocytic elastase together with other lysosomal enzymes, primarily within the lung, might lead to a repeated local proteinase-proteinase inhibitor imbalance [40–42] thus favoring pulmonary disease in long-term RDT patients.

The deleterious release of granulocytic proteinases during hemodialysis is partly counteracted by the diminution of 'unspecific' proteolytic (azocasein-hydrolyzing)

activity accumulated in plasma in the dialysis-free interval. The decrease of this unspecific proteolytic activity during hemodialysis might be caused by the removal of yet unidentified low molecular weight factors. Such factors could stimulate the activation of proenzymes (e.g. plasma prokallikrein) or the release of lysosomal proteinases into the plasma of untreated patients. They might also be responsible for the stimulation of phagocytosis and/or increased protein breakdown within the cells. Characterization of the azocasein-splitting activity using more specific substrates might be a helpful tool in evaluating the origin and nature of this proteolytic effect.

Summarizing our results, we could clearly demonstrate that hemodialysis therapy is still connected with considerable side effects probably due to the contact of white blood cells with the dialyzer cellophane membranes. Improvement of such membranes leading to reduced liberation of proteolytic cell constituents should, therefore, enhance the positive effects of hemodialysis therapy.

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Walter H. Hörl, MD, PhD,
Medizinische Universitätsklinik,
Josef-Schneider-Strasse 2,
D–8700 Würzburg (FRG)