Isolation of Modified Ubiquitin as a Neutrophil Chemotaxis Inhibitor from Uremic Patients

Gerald Cohen, Michael Rudnicki, and Walter H. Hörl
Division of Nephrology, Department of Medicine, University of Vienna, Vienna, Austria.

Abstract. Uremic toxins are factors that accumulate in the serum and peritoneal cavity of uremic patients. They are responsible for a variety of functional disturbances and also contribute to the increased risk of infection by interfering with essential functions of the specific immune response. From the peritoneal effluent of peritoneal dialysis (PD) patients, a peptide was isolated by applying three different chromatographic methods. This peptide inhibits the chemotactic movement of polymorphonuclear leukocytes (PMNL) in an in vitro assay in a concentration-dependent, nonreversible manner, and therefore belongs to the group of uremic toxins. Amino acid sequencing showed that the isolated peptide has the same amino terminal sequence as ubiquitin. The peptide also reacted with anti-ubiquitin antibodies in a Western blot experiment, but had a more acidic isoelectric point than ubiquitin. By using affinity chromatography, anti-ubiquitin antibody binding fractions were isolated from all PD and hemodialysis (HD) patients investigated. These fractions contained the same acidic band and also significantly inhibited PMNL chemotaxis. Ubiquitin per se had no effect on PMNL chemotaxis. Therefore, it is concluded that from PD and HD patients a modified form of ubiquitin was isolated, and this modification was responsible for its inhibitory effect.

One of the major factors causing the high incidence of morbidity and mortality among uremic patients undergoing hemodialysis (HD) and peritoneal dialysis (PD) treatment is the increased risk of bacterial infections (1–3). Between 15 and 20% of all cases of mortality among hemodialysis patients are due to infection (summarized in reference 4). An impairment of the unspecific immune defense system, especially of polymorphonuclear leukocytes (PMNL), is primarily responsible for this situation (5). PMNL are cells of the first-line immune defense and are also the main immune cells in the peritoneal cavity during bacterial infections (6).

A variety of factors contribute to the diminished functions of PMNL found in uremia. Toxic substances that directly interfere with PMNL activities accumulate in the serum of uremic patients and are partly responsible for their susceptibility to bacterial infections. A number of uremic toxins have already been purified and characterized. In our laboratory, a granulocyte inhibiting protein with homology to Ig light chains has been isolated (7). We could show that free Ig light chains per se are able to interfere with essential PMNL functions (8). A granulocyte inhibiting protein with homology to β2-microglobulin was also isolated from hemodialysis patients (9) and from patients undergoing continuous ambulatory peritoneal dialysis (CAPD) treatment (10). Angiogenin was purified from uremic patients as a PMNL degranulation-inhibiting protein (11), and complement factor D was shown to adversely affect PMNL functions (12,13). Furthermore, p-cresol was identified as a uremic solute that impairs the respiratory burst activity of PMNL (14).

In the present study, we describe the isolation of a modified version of ubiquitin from the peritoneal effluent of PD patients and from the ultrafiltrate of high-flux HD patients. We show that the purified protein significantly inhibits PMNL chemotaxis, whereas unmodified ubiquitin has no effect on this essential PMNL function.

Materials and Methods

Isolation of the Chemotaxis-Inhibiting Protein

Patients. The chemotaxis-inhibiting protein (CIP) was originally isolated from the effluent of a patient (patient 1) undergoing CAPD, using three different chromatographic methods. The patient was treated with 2 L of PD solution four times daily. CIP was also isolated by affinity chromatography from the same patient undergoing nightly intermittent PD (NIPD) treatment and from two other CAPD patients (patients 2 and 3) treated in the same way as patient 1. From the ultrafiltrate of three HD patients (patients 4 to 6), CIP was isolated as well. Informed consent was obtained from all patients. Care was taken that the patients did not have any clinical signs or symptoms of infection interfering with our study. The serum C-reactive protein concentration was lower than the detection limit (0.5 mg/dl). The PD effluents were without any laboratory findings responsible for peritoneal infections.

Isolation of CIP by Three Chromatographic Methods

High molecular weight proteins were removed from the CAPD effluent by using a polysulfone membrane with a cutoff of approximately 60 kD (F60; Fresenius, Oberursel, Germany). Then, the CAPD effluent was concentrated about 30-fold, and the NIPD effluent was concentrated about 100-fold with Cuprophan membranes, with a cutoff of approximately 5 kD (Hemoflow E3 or E4, Fresenius). Afterward, the concentrate was chromatographed on a fast-protein...
liquid chromatographic (FPLC) system (Pharmacia LKB Biotechnol-
yogy, Uppsala, Sweden), and the protein was detected with a variable
wavelength monitor (VWM 2141, Pharmacia) at wavelengths of 225
and 214 nm.

**Ion Exchange Chromatography.** In a first step, the concentrate
was applied to FPLC, using an ion exchange Mono Q HR 10/10
column equilibrated with 20 mM Tris-HCl, pH 8.0. The flow rate was
4 ml/min. The biological activity (see below) was found in the
bound material and was concentrated by lyophilization, resus-
pended in water, and dialyzed against 200 mM sodium phosphate
buffer, pH 7.2, using a Spectra/Por molecular-porous membrane
(Spectrum, Houston, TX) with a molecular cutoff of approximately 1
kD. The bound material that was inactive in our in vitro assay was
eluted with 1 M NaCl in 20 mM Tris-HCl, pH 8.0, to regenerate the
column for next use.

**Size Exclusion Chromatography.** The sample was then chromo-
matographed on FPLC with a Superdex 75 prep grad column using
200 mM sodium phosphate buffer, pH 7.2, and a flow rate of 1
ml/min. The protein peak containing the biological activity was in the
molecular weight range of 8 to 10 kD, was concentrated by lyophi-
lization, dissolved in water, and dialyzed against water.

**Reversed-Phase Chromatography.** The material was further
chromatographed on FPLC with a Resource 1-m reversed-phase
chromatography column (Pharmacia), using a gradient from 0 to
100% acetonitrile with 0.05% (vol/vol) trifluoroacetic acid (Aldrich
Chemical Co., Milwaukee, WI) with a corresponding gradient of
water from 100 to 0%. The flow rate was 4 ml/min. Peak fractions
have been extensively dialyzed against phosphate-buffered saline
(PBS; pH 7.2). The fraction that had been eluted at 30% acetonitrile
showed the highest biological activity and was used for further in-
vestigations.

**Isolation of CIP by Immunoaffinity Chromatography**
Concentrates of the PD effluents were prepared as described above.
After identification by amino acid sequencing of CIP as being homol-
ogous to ubiquitin, concentrates of the PD effluents that were prepared
as described above and ultrafiltrates obtained from HD patients were
passed through an immunoaffinity column specific for ubiquitin.
For the preparation of this column, anti-ubiquitin antiserum (U-5379,
Sigma) was coupled onto a N-hydroxysuccinimide-activated HiTrap
affinity column (Pharmacia), according to the manufacturer’s instruc-
tions. After washing the column with PBS, the bound material was
eluted by lowering the pH (0.2 M Glycine-HCl, pH 2.8). The fractions
were neutralized immediately after elution by a 1/5 vol of 1 M
Tris-HCl, pH 8.0. The protein-containing fractions were identified by
the bicinchoninic acid protein assay (Pierce, Rockford, IL). They were
pooled, dialyzed against PBS diluted with water 1 to 40, lyophilized,
and then taken up in water in a 40th of the original volume so that
the sample was finally dissolved in 1 × PBS.

**Characterization of CIP**

**Molecular Characterization.** Sodium dodecyl sulfate-polyac-
rylamide gel electrophoresis (SDS-PAGE), 8 to 25% gels, or high-
density gels and isoelectric focusing (pl range, 3 to 9) was performed
using the Phast System (Pharmacia). For the reducing SDS-PAGE,
10% (vol/vol) beta-mercaptoethanol was added to the sample buffer,
and the sample was boiled for 3 min before loading. The reactivity of
CIP with an anti-ubiquitin antibody was tested by Western blotting.
After electrotransfer of the proteins from the SDS polyacrylamide gels
to a nitrocellulose membrane, the transferred proteins were detected
with an anti-ubiquitin antiserum (U-5379, Sigma), horseradish perox-
idase-labeled goat-anti-rabbit antibodies, and the enhanced chemilu-
minescence detection system (Amersham International, Little Chal-
font, Buckinghamshire, United Kingdom). The amino acid sequence
was determined by automatic Edman degradation (model 476A; Ap-
plied Biosystems, Foster City, CA) in the protein chemistry laboratory
of the Institute for Biochemistry of the University of Vienna by Dr. R.
Prohaska.

**PMNL Isolation**
PMNL were isolated as described previously (15). Briefly, 10 ml of
the venous whole blood of healthy donors was anticoagulated with 10 
μl of heparin sodium (Liquemine Roche iv; Hoffmann-Roche, Basel, Swit-
zerland) and put on top of 10 ml of Ficoll-Paque (Pharmacia). After
approximately 45 min, most of the erythrocytes sedimented into the
Ficoll layer. A Percoll (Pharmacia) step gradient (63% vol/vol and 72%
vol/vol) was used to separate granulocytes, lymphocytes, monocytes,
and thrombocytes that stayed in the plasma supernatant. The PMNL band
was isolated, the PMNL were washed twice in PBS, and the cells counted in a
cell counter (Cell Dyn 610, Abbott, Wiesbaden, Germany). The viability
of the PMNL obtained by this protocol was greater than 95% as
determined by the trypan blue (Sigma) exclusion assay.

**Chemotaxis Assay**
We used the under-agarose method (16) to test the inhibitory effect
of our fractions or of the purified factor on PMNL chemotaxis. PMNL
were resuspended in PBS at a concentration of 1 × 10^6 cells/10 μl in
PBS or in PBS containing the isolated peptide at a final concentration
of 20 μg/ml and incubated for 15 min at 37°C before starting the
chemotaxis assay. N-formyl-methionyl-leucyl-phenylalanine (Sigma),
dissolved in Hank’s buffer with Ca^2+ and Mg^2+ (SeroMed, Bio-
chrom, Berlin, Germany), was used as a chemoattractant at a concen-
tration of 2.5 × 10^-7 M. Afterward, the agarose plates were incubated
for 100 min at 37°C. Then the cells were fixed with methanol and
paraformaldehyde and stained with Giemsa (Merck, Darmstadt, Ger-
many). The distance the cells migrated under the agarose was mea-
sured under the microscope. For each isolate, between eight and 12
independent assays were performed. The results were statistically
evaluated by paired analyses.

**Results**

**Initial Isolation and Characterization of CIP**
We concentrated the peritoneal effluent obtained from pa-
tient 1 undergoing CAPD treatment, as described in Material and
Methods. To obtain a pure substance that shows chemo-
taxis inhibiting activity, we used three different chromatographic methods (see Material and Methods). After each purifi-
cation step, i.e., ion exchange chromatography, gel filtration,
and reversed-phase chromatography, we controlled the biolog-
ical activity of the individual fractions in our in vitro chemotaxis assay. Furthermore, we checked the purity of the most active fractions by nonreducing SDS-PAGE. Figure 1A shows the progress of purification using the concentrated CAPD effluent of patient 1, resulting in the pure protein with chemotaxis inhibiting activity. Reducing (Figure 1B, lane a) and nonreducing (Figure 1B, lane b) SDS-PAGE of CIP show a single band, indicating that we obtained a pure peptide that does not consist of subunits connected by disulfide bridges.

The sequence of the 16 N-terminal amino acids of CIP is Met-Gln-Ile-Phe-Val-Lys-Thr-Leu-Thr-Gly-Lys-Thr-Ile-Thr-
Leu-Glu. This is exactly the same primary structure as found at the N terminus of ubiquitin (17).

Nonreducing SDS-PAGE of commercially available ubiquitin (Figure 2, lane a) and of CIP (Figure 2, lane b) shows that both peptides comigrate, and therefore have about the same size. The molecular mass of ubiquitin predicted from its amino acid sequence is 8.5 kD (17). The anomalous migration of ubiquitin as a 5.5-kD band on the SDS polyacrylamide gel indicates the incomplete unfolding of the polypeptide chain. This finding is consistent with the highly globular compact conformation of ubiquitin (18). In a Western blotting experiment (Figure 1C), we found that CIP is able to react with anti-ubiquitin antibodies. Isoelectric focusing shows that CIP has an isoelectric point of 5.2 (Figure 3, lane a). This is much more acidic than the isoelectric point of unmodified ubiquitin of approximately 7 (Figure 3, lane b) (19).

Inhibitory Effect of CIP on PMNL Chemotaxis

Figure 4 shows that CIP significantly inhibited the chemotactic movement of PMNL from healthy donors. On the other hand, commercially available unmodified ubiquitin had no effect. Furthermore, we showed that the inhibitory effect of CIP was not reversible (Figure 4). Nevertheless, the viability of PMNL was not influenced in the presence of CIP, as determined by the trypan blue exclusion assay (data not shown). We also found that CIP inhibits PMNL chemotaxis in a concentration-dependent manner (Table 1). We tested the ability of CIP to attract normal PMNL. This was not the case within the concentration range tested (2.5 $\times$ 10^{-6} M to 2.5 $\times$ 10^{-8} M) (data not shown).

Affinity Chromatography and Characterization of Anti-Ubiquitin Antibody Binding Fractions

Our data suggested that we isolated a modified form of ubiquitin from the peritoneal effluent and that this modification is responsible for the inhibiting effect on PMNL chemotaxis. In a second set of experiments, we prepared an affinity column specific for ubiquitin and applied the peritoneal effluent obtained from the same patient (at this time undergoing NIPD...
Chemotaxis-Inhibiting Activity of Anti-Ubiquitin Antibody Binding Fractions

We tested all fractions obtained by immunopurification in our in vitro chemotaxis assay. Figure 5 shows that the samples obtained from all PD patients and from all HD patients significantly inhibited the chemotactic movement of PMNL.

Discussion

In the present study, we isolated from PD and HD patients a peptide with homology to ubiquitin and showed that it has the ability to inhibit the chemotactic movement of PMNL in a concentration-dependent manner.

Uremic patients have an increased risk of infection, and as a result they have a high incidence of morbidity and mortality (20). In fact, despite intensive research and improved clinical conditions within the past 20 years, bacterial infections are still one of the leading causes of death in patients with chronic renal failure. Because PMNL play a key role in the unspecific immune defense against bacterial infections, the function of PMNL isolated from uremic patients has been studied intensively.

These investigations of the PMNL-modulating activities of uremic toxins are an important contribution to a better understanding of the immune status of patients with chronic renal failure and to future applications in diagnosis and therapy. The PD effluent represents an important starting material for the isolation of uremic toxins. The fact that many disturbed functions of PMNL can be corrected by PD treatment is an indication for the presence of uremic toxins in the peritoneal cavity of PD patients. Furthermore, it was shown that addition of CAPD effluents to PMNL of healthy donors markedly depressed phagocytosis (21) and also interfered with their ability to kill ingested Staphylococcus epidermidis (22).

To have a uremic toxin, the compound should be retained in the uremic serum, and the concentration should be higher in the uremic than in the normal plasma. Although the majority of proteins that appear in peritoneal effluent come from the vascular compartment, it is possible that some are generated from...
cells within the peritoneal cavity or within the peritoneal membrane. We therefore looked up this component in uremic serum ultrafiltrate to exclude that CIP is only extracted from peritoneal effluent and produced in the peritoneum. This study provides evidence that the protein is present in the blood of uremic patients. The adequate removal of uremic solutes in the higher molecular weight range during high-efficiency and high-flux hemodialysis has been studied (23).

An obvious consequence of the partial removal of PMNL-inhibiting factors from the uremic serum by PD treatment and consequently their presence in the peritoneal cavity is that these toxins exert a negative influence on functions of peritoneal PMNL. Whereas most of the cells within the peritoneal cavity of a PD patient are macrophages in a stable situation, after the onset peritonitis that is accompanied by a massive and rapid influx of cells into the peritoneal cavity, more than 85% of the cells are PMNL (6). Therefore, uremic toxins affecting PMNL functions seem to be at least partly responsible for the high susceptibility of PD patients to peritonitis and, hence, for the increased risk of morbidity and mortality in this group of patients.

Chemotaxis of PMNL is the first important step of the unspecific immune response (24). Whereas the transendothelial migration of PMNL is mainly regulated by interleukin-8 (25), N-formyl-methionyl peptides that are generated by the invading microorganisms are the main chemotactants in bacterial infections and induce PMNL finally to move to the site of infection (26). Therefore, we used the peptide N-formyl-methionyl-leucyl-phenylalanine, one of the most potent within this group of chemotactants (27), in our in vitro chemotaxis assay.

We demonstrated that CIP significantly inhibits PMNL chemotaxis (Figure 4). Because it has been described (28) that PD treatment per se might induce the generation of a chemotractant, we tested this ability. However, CIP did not attract normal PMNL.

Sequencing of the N terminus showed that the first 16 amino acids of CIP are identical to those of ubiquitin. Ubiquitin is a polypeptide with a molecular weight of approximately 8.5 kD. Its 76 amino acids are highly conserved in evolution. In our control experiments, we used bovine ubiquitin that has the same amino acid sequence as human ubiquitin (29). Ubiquitin is present in all cells investigated so far, i.e., in bacteria, yeast, higher plants, and animals (30). It can be attached in an ATP-requiring process to other proteins via an isopeptidic linkage between its C-terminal carboxyl group and the epsilon-amino groups of lysine residues in the target protein. This posttranslational protein modification has a variety of biological effects. The most prominent function is the participation of ubiquitin in the proteosomal protein degradation (31): Ubiquitin covalently bound to proteins can target them to a complex protease called the 26S proteasome that degrades misfolded proteins within the cell. Usually, the formation of ubiquitin oligomers is necessary for proteasome binding and subsequent protein destruction (32). The fact that we isolated by affinity chromatography only material that migrates as a single band in SDS-PAGE (Figure 2) at the same distance as ubiquitin indicated that the anti-ubiquitin antibody-binding fractions contained only ubiquitin and/or a modified form of it, but no ubiquitin oligomers or other proteins with ubiquitin covalently attached to them.

The increased protein degradation leading to muscle wasting in uremia is caused by metabolic acidosis induced ATP- and ubiquitin-dependent proteolysis (33,34). Furthermore, the ubiquitination of short-living regulatory proteins plays an important role in modulating their intracellular concentrations. Besides those intracellular functions of ubiquitin, excreted ubiquitin was isolated from the culture supernatant of oocytes and shown to inhibit the cytotoxic properties of platelets (35). A significantly increased level of ubiquitin was described in the plasma of uremic patients and patients undergoing hemodialysis treatment (36). At present, we cannot give exact in vivo concentrations of the modified ubiquitin in uremic patients.

CIP has about the same size as ubiquitin as shown by SDS-PAGE (Figure 2), but has a more acidic isoelectric point of 5.2. Because ubiquitin did not show any chemotaxis inhibitory activity, we conclude that CIP is a modified form of ubiquitin and that this modification is responsible for its biological activity. Two types of modifications, i.e., carboxymylation and glycation, could explain our findings, because both reactions lead to a loss of positive charge by modifying lysine residues in proteins. The molecular weight change caused by either modification would not necessarily be big enough to be detected by SDS-PAGE.

A small percentage (approximately 0.8%) of urea that accumulates in the serum of uremic patients is converted spontaneously to cyanate (37). Cyanate can react with the epsilon-amino groups of lysins, leading to a carbamoylated protein (38). Glucose can react nonenzymatically with lysine residues, leading to Schiff-base intermediates that react to stable Amadori products, the glycated proteins (39). There also are experiments indicating that soluble proteins within the peritoneal cavity can be modified by glycation during CAPD treatment. This seems not to be the case for CIP, because we could isolate CIP from the ultrafiltrate of HD patients as well. Furthermore, it could be shown that glycated proteins preferentially accumulate in the CAPD effluent (40). Experiments to identify the type of modification leading to CIP are ongoing in our laboratory.

We conclude from the results presented here that a modified form of ubiquitin (CIP) exists in the peritoneal cavity of PD patients and that this modification is responsible for the inhibitory effect of CIP on PMNL chemotaxis and, hence, for the increased risk of infection, leading, at least in part, to a higher incidence of morbidity and mortality in this group of patients.

References


