Kidney International, Vol. 49 (1996), pp. 1304–1313

Advanced oxidation protein products as a novel marker of oxidative stress in uremia

VÉRONIQUE WITKO-SARSAT, MIRIAM FRIEDLANDER, CHANTAL CAPEILLÈRE-BLANDIN, THAO NGUYEN-KHOA, ANH THU NGUYEN, JOHANNA ZINGRAFF, PAUL JUNGERS, and BÉATRICE DESCAMPS-LATSCHA

INSERM U25, Department of Biochemistry, INSERM U90, and Department of Nephrology of Necker Hôpital, CNRS URA 400, Paris, France, and Department of Medicine, Case-Western Reserve University, Cleveland, Ohio, USA

Advanced oxidation protein products as a novel marker of oxidative stress in uremia. Evidence suggests an imbalance between antioxidant and oxidant-generating systems resulting in oxidative stress in uremic patients. As plasma proteins are critical targets for oxidants, we developed a novel spectrophotometric assay which allows to detect advanced oxidation protein products (AOPP) in uremic plasma. By size-exclusion chromatography AOPP are retrieved in two distinct peaks at 600 and below 80 kDa in uremic plasma, while no such peaks are found in control plasma. Further biochemical characterization revealed that AOPP are carried by oxidized plasma proteins, especially albumin and do not have oxidant properties. AOPP increased in a dose-dependent manner following *in vitro* exposure of plasma or purified human serum albumin (HSA) to hypochlorous acid. Advanced glycation end products of human serum albumin (AGE-HSA) also increased AOPP levels. *In vivo*, plasma level of AOPP was the highest in patients on hemodialysis, followed by those on peritoneal dialysis and by undialyzed patients with advanced chronic renal failure. AOPP levels correlated with plasma concentrations of dityrosine and AGE-pentosidine, as indices of oxidant-mediated protein damage, but not with thiobarbituric reactive substances as lipid peroxidation markers. A close correlation was also found between AOPP and neopterin levels, suggesting that AOPP could be part in the monocyte-mediated inflammatory disorders associated with uremia. In conclusion, we propose the measurement of AOPP as a reliable marker to estimate the degree of oxidant-mediated protein damage in uremic patients and to predict the potential efficacy of therapeutic strategies aimed at reducing such an oxidative stress.

Chronic renal failure (CRF) is associated with a multifactorial immuno-inflammatory syndrome that occurs early in the course of renal failure, worsens with its progression and culminates on maintenance dialysis therapy, as recently reviewed in [1]. Among the mechanisms involved in such uremia-related immuno-inflammatory disorders, a dysregulation in the balance between pro-inflammatory cytokines and their inhibitors has recently been demonstrated [2, 3]. It has also been proposed that increased production of oxidants by phagocytes, in the face of defective antioxidant defenses, results in chronic oxidative stress that further enhances the inflammatory state [4]. This has been especially well documented in patients on maintenance hemodialysis (HD) in whom blood interaction with bioincompatible membranes triggers circulating neutrophils to produce numerous reactive oxygen species, including superoxide anion, hydrogen peroxide, hydroxyl radical and hypochlorous acid [5–10]. In this setting, the oxidant scavenging potential of plasma components is likely to be overwhelmed [11] as evidenced by a decrease in ascorbic acid [12], superoxide dismutase [13] and the glutathione system [14–16] in both pre-dialysis and HD patients. The resulting oxidative stress could be an important event in the pathogenesis of atherosclerosis [17], which still is the leading cause of morbidity and mortality in uremic patients [18–20].

With respect to the markers of such oxidative stress, some reports have shown that plasma level of malondialdehyde (MDA), a byproduct of lipid peroxidation which reacts with thiobarbituric acid (TBA) and is also referred to as TBA-reacting substances (TBARS), is elevated in both dialyzed and undialyzed CRF patients [16, 21, 22].

In contrast to lipids, the reactions of proteins with various oxidants have not been extensively studied, and it was sometimes assumed that proteins are not particularly susceptible to free radical damage. This assumption has been invalidated in recent years, and it is now clear that amino acids, peptides, and proteins are vulnerable to attacks by a variety of free radicals and related oxidants [23, 24]. Oxidation of particularly sensitive amino acid residues, aggregation, cross linking, fragmentation, as well as loss of enzymatic or other functional properties are but a few of the documented examples. Interestingly, we have previously shown that exposure of β_2-microglobulin to hydroxyl radicals leads to aggregation and dityrosine formation [25], which may be of pathophysiological relevance in view of the β_2-microglobulin amyloidosis observed in dialysis patients [26, 27].

In this study we describe and characterize a novel oxidative stress marker referred to as advanced oxidation protein products (AOPP), which are present at high levels in the plasma of uremic patients.

Methods

Patients

One hundred nineteen patients were enrolled in the study after giving their informed consent. They included 17 undialyzed

Received for publication August 5, 1995
and in revised form December 20, 1995
Accepted for publication December 25, 1995

patients with advanced renal failure defined by creatinine clearance (C_{Cr}) < 20 ml/min (N = 17) and 102 patients receiving dialysis for at least one year, including 25 on chronic ambulatory peritoneal dialysis (CAPD) and 77 on maintenance hemodialysis (HD). Hemodialysis was performed three times weekly; 47 of the HD patients (followed at the Dialysis Center of University Hospital, Cleveland) were treated with hollow-fiber dialyzers equipped with polysulfone (F8 or F80; Fresenius USA, Concord, CA, USA); these hollow-fiber dialyzers were re-used after processing on Renatron I (Renal Systems, Minneapolis, MN, USA) using Renalin®. The other 30 HD patients were treated with single-use dialyzers equipped with polyacrilonitrile AN-69 membranes (Hospal, Meyzieu, France) (N = 15), cellulose acetate membranes (Baxter Laboratories, McGaw Park, IL, USA) (N = 10) or polysulfone membranes (Fresenius, Bad Homburg, Germany) (N = 5). Dialysate was of standard ionic composition, and bicarbonate was used as buffer substrate in all cases. Patients with systemic lupus erythematosus, malignant tumors, or acute infectious illnesses, and those receiving immunosuppressive therapy were excluded from the study. Controls consisted of 41 healthy subjects recruited among blood donors of Necker Hôpital Blood Center.

Blood collection and plasma isolation

Blood was taken from undialyzed patients with advanced renal failure and patients on CAPD at the time of routine laboratory investigations. In HD patients, blood was drawn from the fistula needle just before the start of a hemodialysis session. Blood (5 to 10 ml) was collected in standard sterile polystyrene vacuum tubes with 5 mM EDTA. Following centrifugation (600 g × 10 min) the plasma was stored in 500 μl aliquots at −70°C until use. Assays were carried out on duplicate samples. As the effect of dialysis-induced hemoconcentration on AOPP plasma levels had to be taken into account in the study of the effect of dialysis sessions, AOPP levels were corrected according to the total protein concentration.

Spectral analysis of AOPP

Spectral analysis using a Kontron UVICON 940 was performed on plasma samples diluted 1:200 in 20 mM phosphate buffer pH 7.4. Absorbance spectra in the range 200 to 400 nm were recorded in plasma samples of HD patients and controls. Absorbance at 340 nm in acidic condition was significantly higher in plasma from HD patients than in plasma from controls. This protocol was adapted to a semiautomated method using a microplate reader (Model MR 5000; Dynatech, France), and calibrated with chloramine-T (Sigma Chemical Company, St. Louis, MO, USA) solutions which in the presence of potassium iodide absorb at 340 nm [28]. Two hundred microliters of plasma diluted 1:5 in PBS, or chloramine-T standard solutions (0 to 100 μmol/liter), were placed in each well of a 96-well microtiter plate (Becton Dickinson Labware, Lincoln Park, NJ, USA), followed by 20 μl of acetic acid. Ten microliters of 1.16 M potassium iodide (KI, Sigma) were then added, followed by 20 μl of acetic acid. The absorbance of the reaction mixture was immediately read at 340 nm in a microplate reader against a blank containing 200 μl of PBS, 10 μl of KI and 20 μl of acetic acid. The chloramine-T absorbance at 340 nm was linear within the range of 0 to 100 μmol/liter. AOPP concentrations were expressed in μmol/liter of chloramine-T equivalents.

Reproducibility was assessed on six plasma samples from HD patients covering a wide range of concentrations (70 to 300 μmol/liter). The intra-assay coefficients of variation (CV) of quadruplicates were less than 5% and the inter-assay CV was below 10%. It was also verified that storage of plasma at −70°C for more than six months did not significantly affect AOPP measurement (data not shown).

Biochemical analysis of AOPP

To test whether AOPP have oxidant properties and may therefore be inhibited by oxidant scavengers, ascorbic acid or methionine (range 0.01 to 100 mM) were incubated with plasma for 15 minutes, and tested for AOPP.

Total protein concentrations using the Biuret method [29] were measured on an Hitachi 717 analyzer (Boehringer Mannheim, Meylan, France). Protein precipitation was performed by mixing one volume of trichloro-acetic acid (TCA, Sigma) with one volume of plasma. This mixture was centrifuged at 10,000 g and AOPP determination was performed on the supernatant.

Delipidation was performed by mixing 200 μl of plasma diluted 1/5 with the same volume of chloroform, rotated for two hours at 4°C and centrifuged at 10,000 g for 10 minutes. The lower phase containing chloroform was discarded, while the upper phase containing delipidated plasma was submitted to a second chloroform extraction and AOPP were measured in the delipidated plasma.

Plasma fractionation by size-exclusion chromatography was performed to determine the molecular weights of the molecules involved in the AOPP activity. One milliter of plasma was diluted 1/5 in PBS, 10 mM EDTA, pH 7.4, loaded on a chromatographic column (1.5 × 70 cm) containing the high-performance size exclusion matrix (Bio-Gel A 0.5 m Gel; Bio Rad, Hercules, CA, USA) and eluted at 4°C at a flow rate of 0.2 ml/min, according to manufacturer's instruction. For each fraction, both protein and AOPP levels were evaluated by measuring OD at 280 nm or OD at 340 nm in acidic condition, respectively. The column was calibrated by loading a mixture of proteins of known molecular weights, including thyroglobulin (669 kDa), γ-globulin (150 kDa), albumin (67 kDa) and myoglobin (17.8 kDa), the order of elution of the mixture of proteins from the column being the inverse of their molecular weight.

Polyacrylamide gel electrophoresis (PAGE)

Nondenaturing PAGE (7.5% acrylamide) was performed in the absence of SDS to visualize protein aggregates. Protein concentrations were adjusted to have 20 μg per well in sample buffer (60 mM Tris-HCl pH 6.8, 10% glycerol, 0.05% Bromophenol Blue).

Denaturing sodium dodecyl sulfate (SDS)-PAGE was performed in the presence or absence of β-mercaptoethanol. Protein concentrations were adjusted to have 40 μg per well in sample buffer (60 mM Tris-HCl pH 6.8, 10% glycerol, 0.05% Bromophenol Blue, 2% SDS with or without 1% β-mercaptoethanol). Protein samples were boiled and run on 12.5% SDS-PAGE along with prestained molecular weight markers (Pharmacia, Piscataway, NJ, USA) including phosphorylase B (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa) and soybean trypsin inhibitor (20 kDa). Gel were stained with either Coomassie Brilliant Blue or silver stain (Biorad).

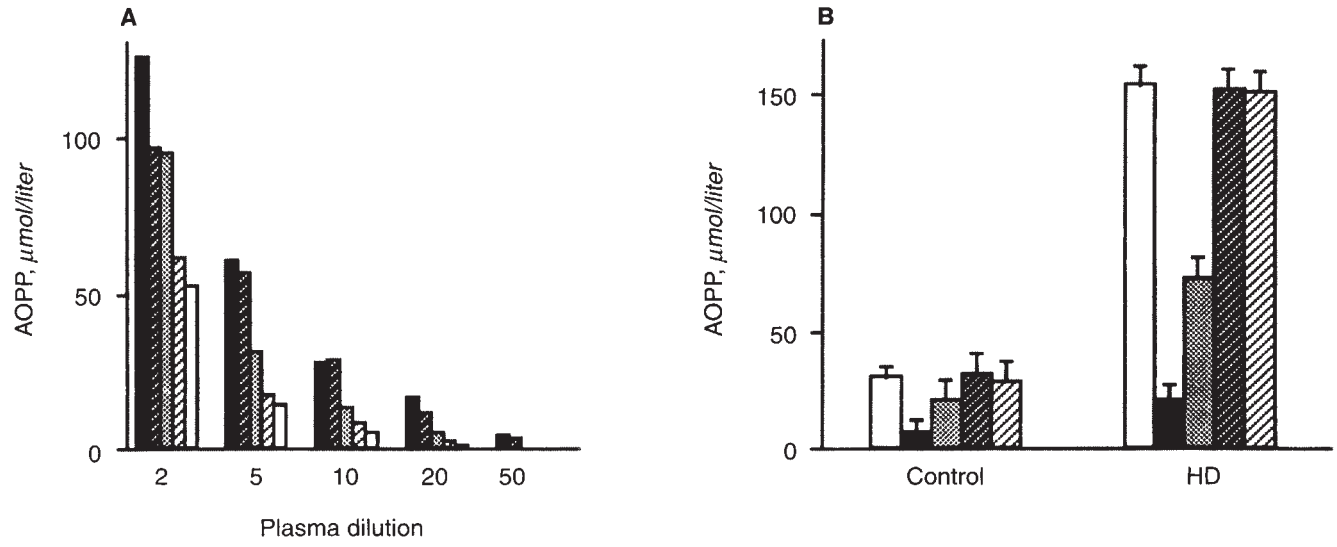

Fig. 1. *Characterization of AOPP measurement.* **A.** Range of linearity of AOPP measurement. Plasma samples from 5 HD patients [(■) HD1, (▨) HD2, (▤) HD3, (▨) HD4, and (□) HD5) were successively diluted 1/2, 1/5, 1/10, 1/20 and 1/50 in PBS and AOPP levels were measured in each dilution. Results are expressed as mean of triplicates. **B.** Effect of plasma TCA deproteinization, chloroform delipidation and oxidant scavengers (100 mM ascorbic acid or 100 mM methionine) on plasma AOPP levels as described in the **Methods** section. Symbols are: (□) no treatment; (■) TCA; (▤) chloroform; (▨) ascorbic acid; (▨) methionine. AOPP levels were measured in plasma samples from control subjects (N = 5) and from HD patients (N = 5) before and after each treatment. Results are expressed as mean ± SEM.

Exposure of proteins to hypochlorous acid

Control plasma or purified human serum albumin (HSA) were exposed to increasing concentrations of HOCl (Fluka Chemika Biochemika, Buchs, Switzerland) ranging from 0 to 50 mmol/liter. HOCl stock solution (100 mM) was freshly prepared in PBS and concentration was measured by spectrophotometry using a molar extinction coefficient of 350 M^{-1} cm^{-1} at 290 nm at pH 12 [30]. In this report, the term HOCl will be used to represent both OCl^- and its protonated form. Various concentrations of HOCl were added to control plasma or to a solution of HSA with concentrations ranging from 0 to 60 mg/ml incubated 30 minutes at room temperature and tested for AOPP.

Measurement of dityrosine

As the formation of dityrosine is an index of oxidant-induced protein cross linking and aggregation [23, 24], we measured dityrosine level in solutions of purified HSA or control plasma exposed to various concentrations of HOCl, and in plasma samples from HD patients. UV and fluorescence spectra are well established for dityrosine, with absorbance peaks at 284 nm (acid) and 315 nm (basic) at pH 7.5, corresponding to an equilibrium between the unionized and ionized forms of dityrosine, and a fluorescence emission maximum at 410 nm for excitation at 315 nm [31]. Protein-bound dityrosine production was assayed by fluorescence measurements after 200-fold dilution of the plasma in 20 mM phosphate buffer pH 7.4 in the presence of 6 M urea [25]. The fluorescence emission of dityrosine generated by the denatured protein sample was recorded from 350 to 450 nm after excitation at 315 nm using a Kontron SF 25 spectrophotometer, and measured at its maximum near 410 nm. The assay was calibrated by means of external standardization using a curve generated in the same urea medium with authentic dityrosine. Its concentration was monitored spectrophotometrically in acid medium using ϵ_{284} = 4.5 $mM^{-1} \cdot cm^{-1}$ [32] and in basic medium using an absorbance ratio A315 nm/A284 nm = 1.50. Dityrosine was synthesized by oxidation of L-tyrosine (Sigma) with horseradish peroxidase (Sigma), and purified using the Anderson method described in [33]. Dityrosine concentrations were normalized to the protein concentration of each sample and expressed in nmoles/mg protein. Intra-assay and inter-assay CV, for a given sample were both below 10%.

Preparation of AGE-HSA and measurement of AGE pentosidine

Non-enzymatic glycation of proteins resulting in advanced glycosylation end-products (AGEs) has been linked to protein oxidation [34]. The AGE-pentosidine, a fluorescent cross link resulting from the reaction of pentoses with proteins [35] was measured as a probe. Plasma protein pentosidine was determined using a modification of the method of Odetti et al [36]. Plasma proteins were precipitated on ice with an equal volume of 10% trichloroacetic acid. The pellets were washed twice with 5% cold trichloroacetic acid and hydrolyzed in 2 ml of 6 N HCl for 16 hours at 110°C in borosilicate tubes with screw caps. Acid was removed by vacuum centrifugation (Savant, Farmington, NY, USA). The hydrolyzed pellet was dissolved in 250 μl 0.01 M heptafluorobutyric acid (Sigma). The equivalent of 4 mg of plasma protein was injected into a high-pressure liquid chromatography (HPLC) system (Waters Division of Millipore, Marlborough, MA, USA). A 25 × 0.46 cm C-18 Vydac type 218TP (10 μm) column was used (Separations Group, Hesperia, CA, USA). HPLC was programmed with a linear gradient of 10 to 17% acetonitrile from 0 to 35 minutes. Pentosidine was eluted at approximately 30 minutes as monitored by fluorescence excitation at 335 nm and emission at 385 nm. Pentosidine prepared according to the method of Sell and Monnier [37] was used as standard. Results were calculated per mg added protein. The intra-assay and

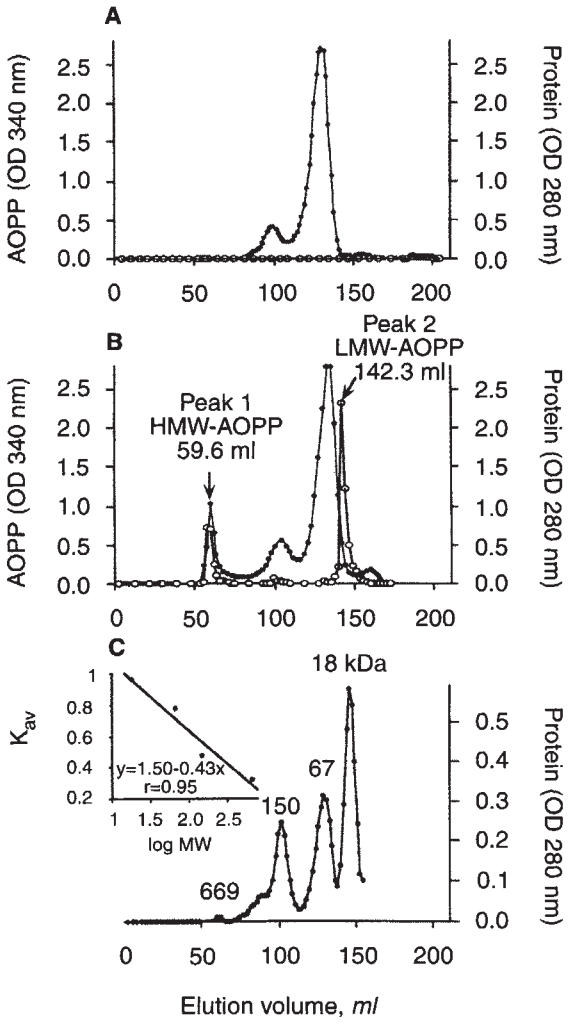

Fig. 2. *Determination of AOPP molecular weights by size-exclusion chromatography.* (**A**) Fractionation of plasma from a control subject (C1) and (**B**) a HD patient (HD1). On each fraction, AOPP was assessed by OD at 340 nm under acidic conditions (○) and protein concentration was assessed by OD at 280 nm (●). **C.** Elution profile of the molecular weight standards including thyroglobulin (669 kDa), γ-globulin (150 kDa), albumin (67 kDa) and myoglobin (18 kDa). Elution volume and OD at 280 nm were measured for each collected fraction. For each standard protein, a partition coefficient, designated Kav, was calculated as follows: Kav = (Ve − V0)/(Vt − V0) where Ve is the elution volume of each component, V0 (58 ml), the void volume, and Vt (109 ml) is the total volume. A semilogarithmic plot of the partition coefficient on molecular weight constitutes the linear standard curve. Fractionation was performed on 3 HD plama samples and compared with 3 controls.

inter-assay CVs for a pentosidine standard of 15 pmol were 11 and 15%, respectively.

AGE-modified HSA (AGE-HSA) was prepared by incubating HSA (50 mg/ml) in the presence of 500 mM glucose in PBS for 65 days at 37°C under sterile conditions. The resulting pentosidine concentrations were 9 and 105 pmol/mg for HSA and AGE-HSA, respectively.

Evaluation of lipid peroxidation by TBARS

Fluorimetric measurement of TBARS was carried out using a commercially available kit according to the manufacturer's instruction (Sobioda, Grenoble, France) [38]. Briefly, tetraethoxypropane (TEP) was used as standard. Hydrolysis of each molecule of TEP yields one molecule of malondialdehyde under the assay conditions. Plasma (100 μl) was mixed with thiobarbituric and perchloric acids, boiled for one hour, and butanol (Farmitalia Carlo Erba, Milan, Italy) was added. The mixture was then vortexed and centrifuged in order to extract MDA. Fluorimetric measurements (excitation at 532 nm and emission at 553 nm) were performed on supernatants using a Kontron SFM 25.

Measurement of C-reactive protein (CRP)

Plasma concentration of CRP, an acute phase protein considered as a non specific marker of inflammatory processes, was determined by immunoturbidimetry according to manufacturer's instruction (Orion Diagnostica, Asnières, France) on an Hitachi 717 analyzer (Boehringer Mannheim, Meylan, France).

Measurement of neopterin

Neopterin, a marker of monocyte-macrophage activation [39] was measured using a commercial RIA according to manufacturer's intruction (Behring Diagnostic, Rueil-Malmaison, France).

Statistical analysis

Statistical analysis was performed using the Statistica software package (Statsoft, Tulsa, OK, USA). Analysis of variance (ANOVA) or Student's *t*-test, paired or unpaired as appropriate, were used to detect differences between the various groups of patients. Simple regression analysis and Pearson *r* correlation coefficient were used to determine the relationships between AOPP and dityrosine, AGE-pentosidine, TBARS, CRP or neopterin levels. Coefficients of variation (CV) were calculated as (standard deviation/mean) × 100. Data are expressed as mean ± SEM.

Results

Biochemical characterization of AOPP measured in plasma

AOPP levels were significantly higher in plasma of HD patients ($N = 10$) than of controls ($N = 10$; 137.6 ± 11.1 vs. 29.4 ± 4.9 μmol/liter, $P < 0.001$). The range of linearity of AOPP measurements was evaluated by testing the same plasma sample at various dilutions. Data obtained in five plasma samples from HD patients indicated linearity up to the dilution 1/20 (Fig. 1A). Incubation of plasma in the presence of an oxidant scavenger such as ascorbic acid or methionine (up to 100 mM) did not modify AOPP values, thus showing that AOPP have no oxidant properties (Fig. 1B).

No significant difference in plasma protein concentration was observed between controls ($N = 10$) and HD patients ($N = 20$), 67.1 ± 1.06 versus 66.9 ± 1.31 g/liter and no correlation between AOPP and plasma protein concentration was observed. No AOPP were retrieved in the supernatant after TCA treatment (Fig. 1B), whereas delipidation of plasma by chloroform extraction halved the AOPP level in HD patients.

Elution patterns obtained after plasma fractionation by size-exclusion chromatography differed between HD and control subjects. As shown in Figure 2B, in plasma from HD patients a peak of protein was detected in the range of high molecular weights, around 670 kDa (Fig. 2C), whereas no such protein peak could be detected in control plasma (Fig. 2A). Interestingly, in plasma from HD patient, this protein peak corresponded to a

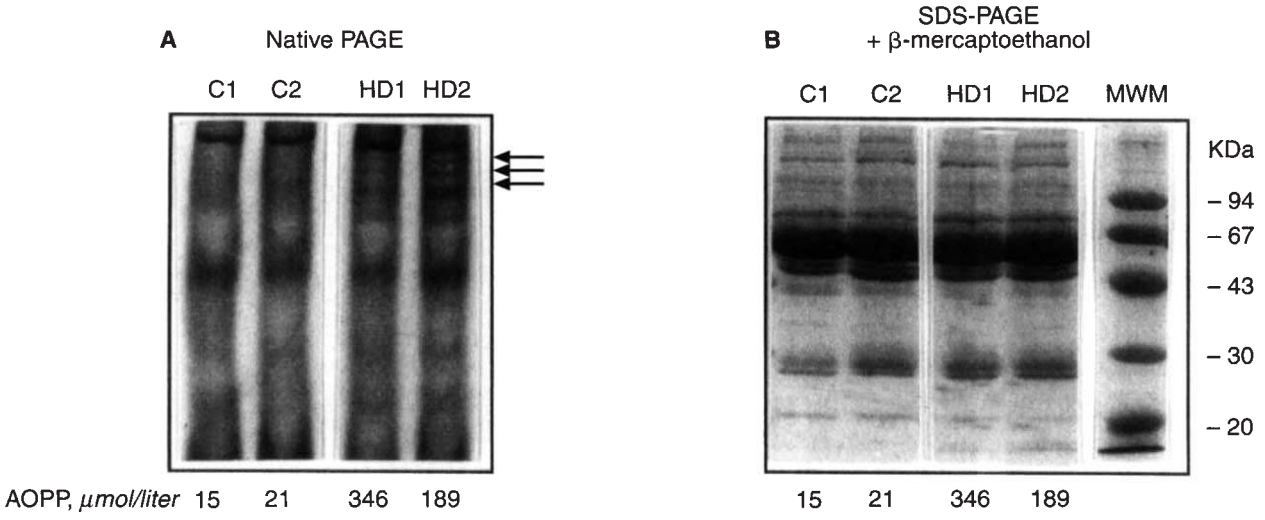

Fig. 3. *Electrophoresis of plasma samples from HD patients as compared to controls.* **A.** PAGE performed under native conditions without SDS or β-mercaptoethanol of two control samples (C1 and C2) and on two plasma samples from HD patients (HD1 and HD2). Arrows indicate protein aggregates in the region of high molecular weights. Corresponding AOPP levels are reported at the bottom of each lane. **B.** PAGE performed under denaturing conditions in the presence of SDS and β-mercaptoethanol. The same plasma samples (C1, C2, HD1 and HD2) were run along with standard molecular weight markers (MWM).

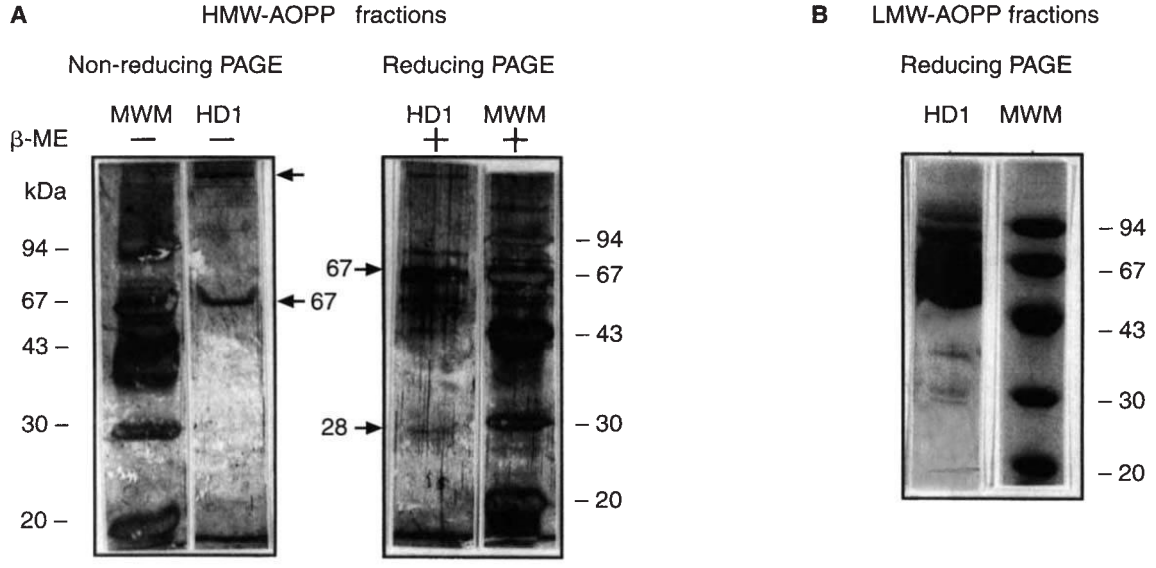

Fig. 4. *Electrophoresis of the AOPP positive fractions obtained by size exclusion chromatography.* **A.** Denaturing PAGE of the fraction containing high molecular weight AOPP (HMW-AOPP). PAGE was performed on the fraction number 27 eluted at 59.6 ml (see Fig. 2B). PAGE was performed in the absence of β-mercaptoethanol (on the left) showing a single prominent band at 67 kDa, or in the presence of β-mercaptoethanol (on the right) showing several bands in addition of the 67 kDa band. **B.** Denaturing PAGE of the fraction containing low molecular weight AOPP (LMW-AOPP). PAGE was performed on the fraction number 64 eluted at 142.3 ml (see Fig. 2B) in the presence of β-mercaptoethanol showing several bands including the prominent at 67 kDa. This electrophoresis has been performed on the fraction obtained with the plasma HD1 and similar results were obtained with the plasma HD2.

peak of AOPP. A second peak of AOPP was detected in HD plasma at a lower molecular weight (around 50 kDa) that partly overlapped the albumin protein peak at 280 nm. No significant AOPP peak could be detected in control plasma.

Native PAGE, which is supposed to preserve the multimolecular structure of proteins, performed on plasma from controls or from dialyzed patients permits the visualization of high molecular weight species in HD patients (Fig. 3A). In contrast, under denaturing condition, no difference was observed between HD and control plasma (Fig. 3B). PAGE together with plasma fractionation suggested that plasma from HD patients exhibit high molecular weight proteins matching the AOPP peak, which may be related to protein aggregates. The fraction corresponding to the high molecular weight AOPP peak (HMW-AOPP fraction number 27 at an elution volume of 59.65 ml, marked with an arrow on Fig. 2B) contained 1.7 mg/ml protein and treatment with

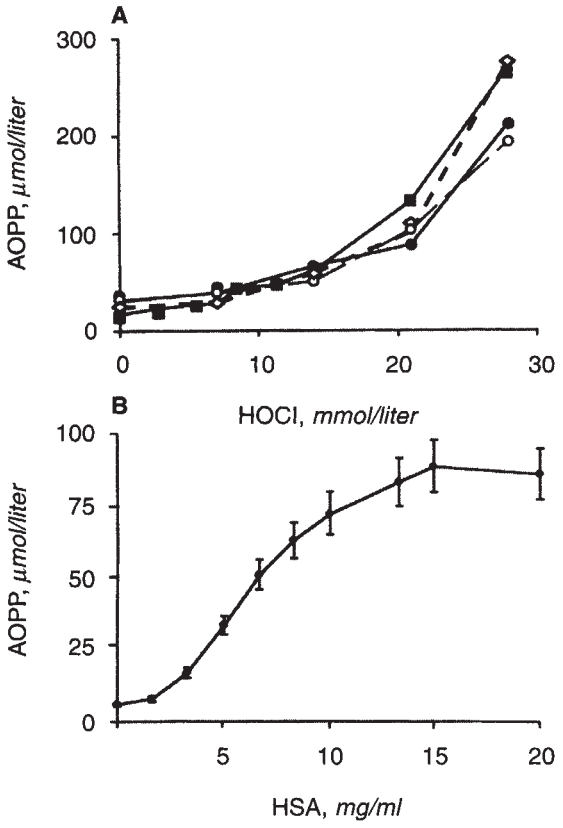

Fig. 5. *Effect of HOCl exposure on AOPP measured in purified HSA solution or in plasma from control subjects.* **A.** Increasing concentrations of HOCl were added either to a purified HSA solution (--◇--; 60 mg/ml) or to control plasma [(—■—) Control 1 and (—●—) Control 2] or to chloroform-delipidated plasma [—○— control 2 + chloroform]. **B.** Increasing concentration of HSA were exposed to 23 mM HOCl. AOPP measurements were performed in quadriplicate. Results (mean ± SEM) are plotted on the curve.

chloroform decreased the AOPP level by 50%, thus suggesting that lipoproteins could be part of this aggregate. However, no lipid peroxidation products, as measured by TBARS, could be detected (data not shown).

PAGE performed on the fraction corresponding to the HMW-AOPP peak, in the presence of SDS to solubilize and dissociate protein aggregates, revealed a prominent band at 67 kDa (likely albumin) as well as a band in the high molecular weight region that did not migrate (Fig. 4A). The same fraction submitted to SDS-PAGE in the presence of a reducing agent such as β-mercaptoethanol, which can reduce disulfide bonds, showed a less intense band in the high molecular weight region and revealed additional bands at 78, 67 and 28 kDa that may correspond to degradation products of oxidized albumin (Fig. 4B). Other bands at 59 and 49 kDa were present but were also observed in the molecular weight markers and therefore could be artifacts due to β-mercaptoethanol, as we previously observed [25].

The fraction corresponding to the low molecular weight AOPP fraction (LMW-AOPP fraction number 64 at an elution volume of 142.3 ml, marked with an arrow on Fig. 2B) had a protein concentration of 0.87 mg/ml and did not appear to contain lipoproteins because chloroform treatment did not modify AOPP and TBARS were undetectable (data not shown). SDS-PAGE electrophoresis performed in the presence of β-mercaptoethanol showed an intense band at 67 kDa, likely albumin (Fig. 4B). Taking into account the poor resolution in the range of molecular weights below 50 kDa with the gel filtration we used, and the high concentration of albumin in plasma, it was not surprising that the LMW-AOPP fraction contained albumin. The contribution of albumin to AOPP will be further discussed.

Hypochlorous acid treatment increases AOPP in purified HSA or plasma from controls

To provide some insights into the *in vivo* mechanism of formation of AOPP we measured AOPP in control plasma samples submitted to increasing concentrations of HOCl, a strong oxidizing agent. We observed that AOPP level increased in a dose-dependent manner with HOCl exposure; the same was observed with delipidated plasma samples (Fig. 5A).

Interestingly enough, similar results were obtained when a solution of HSA (60 mg/ml), close to the physiologic albumin plasma concentration, was exposed to increasing concentrations of HOCl (Fig. 5A). AOPP formation also increased with albumin concentration (Fig. 5B).

Relationships between dityrosine and AOPP

In vitro hypochlorous acid treatment induces dityrosine formation in purified HSA or in plasma proteins from controls (Fig. 6A). *In vivo*, in plasma from HD patients, the excitation spectra of the 410 nm emission consisted of two bands centered at 317 nm and near 285 nm (Fig. 6B), these wavelengths matching those of the absorption bands of dityrosine at 315 and 284 nm. In contrast, control samples were devoid of the 317 nm band characteristic of ionized dityrosine. Dityrosine plasma levels were significantly higher in HD patients ($N = 20$) than in controls ($N = 10$; 1.03 ± 0.12 vs. 0.36 ± 0.05 nmol/mg protein, $P = 0.004$, Fig. 6C). Moreover, a significant correlation between AOPP and dityrosine levels was observed *in vivo* ($r = 0.63$, $P < 0.001$, Fig. 6D).

Relationship between advanced glycation end products (AGE) and AOPP

Because AGE formation is linked to oxidative stress, we measured AOPP levels in a solution of purified HSA as compared to AGE-HSA and HOCl-treated HSA, all protein concentrations being adjusted at 10 mg/ml (Fig. 7A). We observed an increase in AOPP levels in both AGE-HSA and HOCl-treated HSA, thus showing that 14 mM HOCl treatment or *in vitro* protein glycation were as effective to induce AOPP formation.

In vivo, AGE-pentosidine levels were higher in HD patients ($N = 47$) than in controls ($N = 7$; 20.5 ± 1.40 vs. 1.73 ± 0.20 pmol/mg protein, $P < 0.001$, Fig. 7B). A highly significant correlation was observed between AGE-pentosidine and AOPP levels ($r = 0.49$, $P < 0.001$, Fig. 7C).

Relationships between AOPP and TBARS

As expected, TBARS levels were significantly increased in HD patients ($N = 42$) as compared to controls ($N = 20$; 5.33 ± 0.18 vs. 3.61 ± 0.11 μm/liter, $P < 0.001$), but no correlation was observed between AOPP and TBARS, thus showing that AOPP are not directly related to lipid peroxidation.

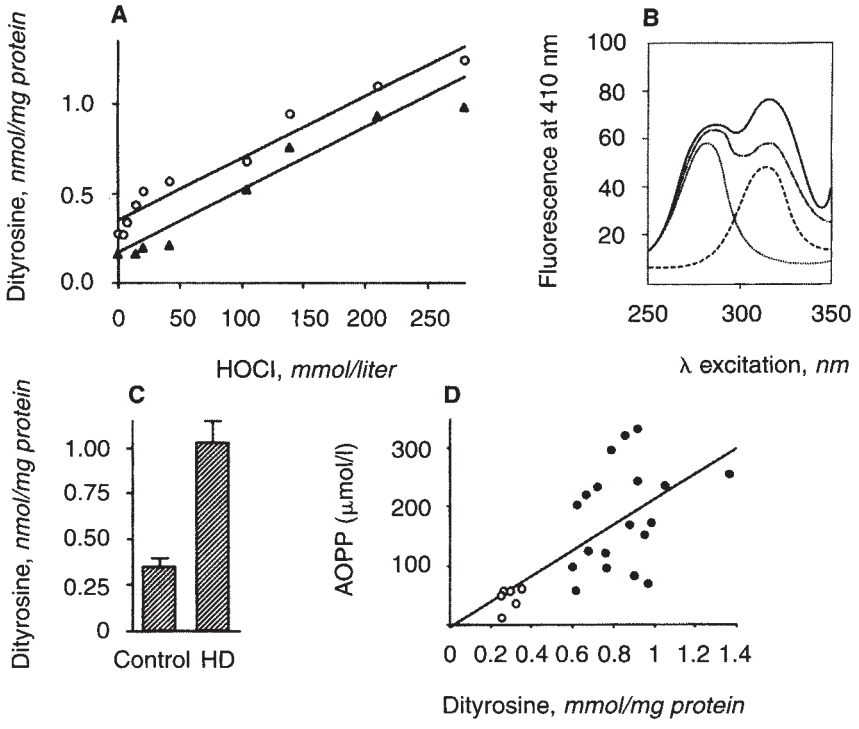

Fig. 6. *Relationship between dityrosine and AOPP measurements.* **A.** Increasing concentrations of HOCl were added either to control plasma (○, $r = 0.98$) or to a purified HSA solution (60 mg/ml; ▲; $r = 0.93$) and dityrosine levels were measured. **B.** Plasma samples were diluted 200-fold in a urea phosphate buffer, pH 7.4, and excitation spectra of the emission band were recorded. Plasma from HD patients (HD1 and HD2) showed two peaks centered at 285 and 317 nm, characteristics of the absorbtion bands of dityrosine. In contrast, the peak at 317 nm is missing in plasma sample from control subject. Symbols are: (——) HD1; (–·–) HD2; (····) Control; (– – –) 0.6 μM dityrosine. Standard dityrosine spectra were recorded as a positive control. **C.** Plasma levels of dityrosine (mean ± SEM) in plasma from controls ($N = 10$) and HD patients ($N = 20$). **D.** Regression analysis between *in vivo* plasma levels of AOPP and dityrosine ($r = 0.63$; $P = 0.001$).

Respective influence of uremia and dialysis procedure on AOPP levels

To evaluate the influence of the dialysis procedure on AOPP levels, we measured AOPP in undialyzed patients with advanced CRF compared to patients treated by CAPD or HD (Fig. 8). AOPP levels were significantly higher in undialyzed patients with advanced CRF (69.4 ± 6.6 μmol/liter, $N = 17$), and even more in CAPD patients (127.9 ± 15.3 μmol/liter, $N = 25$) than in controls (33.9 ± 3.4 μmol/liter, $N = 41$; $P < 0.01$), and were even higher in HD patients (163.7 ± 8.4 μmol/liter, $N = 77$, $P < 0.001$), thus indicating that AOPP level increases in uremia and is further enhanced by maintenance dialysis. Of note, AOPP levels were not influenced by diabetes mellitus, as no difference between diabetics and non-diabetics was found in CAPD or HD patients.

In the group of HD patients treated in the same center (Necker Hôpital) with single-use high-flux dialyzers, AOPP levels at start of hemodialysis sessions were not significantly influenced by the nature of the dialysis membrane [153.4 ± 19.1 μmol/liter ($N = 5$), 195.2 ± 24.2 μmol/liter ($N = 10$) and 140.8 ± 17.9 μmol/liter ($N = 15$), respectively for cellulose acetate, polysulfone, and polyacrilonitrile AN69 membranes]. AOPP levels measured at the beginning and at the end of a single dialysis session in 10 patients treated with polysulfone membranes were 223.6 ± 19.8 versus 267.5 ± 16.5 μmol/liter ($P < 0.05$).

Relationships between AOPP and CRP or neopterin

Plasma levels of CRP were significantly higher in plasma from HD patients ($N = 44$) than from controls ($N = 10$; 24.2 ± 4.2 vs. 1.7 ± 0.67 mg/ml, $P < 0.001$). However, no correlation was observed between AOPP and CRP levels ($r = 0.28$, $P = 0.12$).

Plasma levels of neopterin, a marker of monocyte activation, were significantly elevated in patients with advanced CRF (32 ± 4 nmol/liter, $N = 17$), on CAPD (82 ± 4 nmol/liter, $N = 25$) or on HD (149 ± 14 nmol/liter, $N = 47$) as compared to controls (1.5 ± 0.5 nmol/liter, $N = 41$; $P < 0.001$ for all). In uremic patients, there was a highly significant correlation between neopterin and AOPP levels ($r = 0.59$, $P < 0.001$; Fig. 9). Interestingly, such a correlation was not found between neopterin and TBARS or neopterin and CRP.

The possibility that the correlation between AOPP and neopterin was only artifactual, in particular due to the absorbance of neopterin at 340 nm, was ruled out by an *in vitro* experiment showing that increasing concentrations of neopterin (up to 20-fold the level found in HD plasma) added to normal or uremic plasma did not increase AOPP concentrations (data not shown).

Discussion

The present study describes a novel oxidative stress marker, referred to as advanced oxidation protein products (AOPP) and its possible clinical relevance in chronic uremia. *In vitro*, AOPP can be formed in purified HSA solution as well as in control plasma following HOCl exposure. *In vivo*, plasma levels of AOPP closely correlate with levels of dityrosine, a hallmark of oxidized proteins, and with pentosidine, a marker of enzymatic protein glycation tightly related to oxidative stress. We thus afford evidence for the first time that oxidant-mediated protein damage occurs in plasma from uremic patients. Plasma levels of AOPP were the highest in patients on hemodialysis, followed by those on peritoneal dialysis. Undialyzed patients with advanced chronic renal failure had lower levels than those on dialysis but markedly higher than healthy controls. We further demonstrated that in undialyzed CRF patients, AOPP level is in close relationship with plasma concentration of neopterin, a marker of monocyte activation.

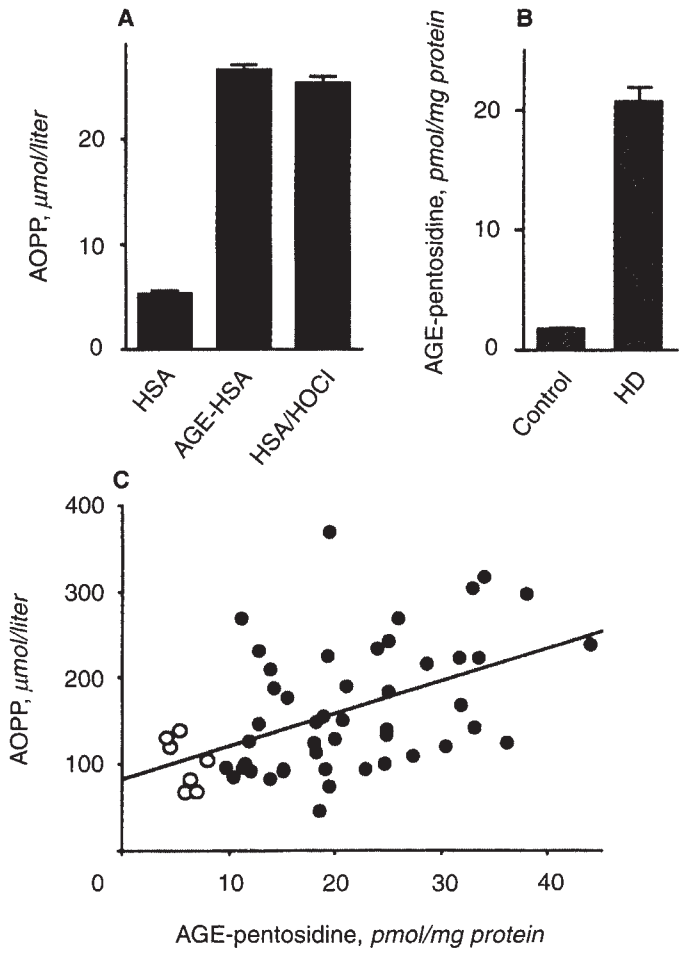

Fig. 7. *Relationship between AOPP and AGE.* **A.** AOPP levels were measured in HSA (10 mg/ml), or in AGE-HSA (10 mg/ml) and in HSA (10 mg/ml) exposed to 23 mM HOCl. **B.** Plasma levels of AGE-pentosidine in controls ($N = 7$) and HD patients ($N = 47$). **C.** Regression analysis between *in vivo* plasma levels of AOPP and AGE-pentosidine ($r = 0.49$; $P = 0.002$).

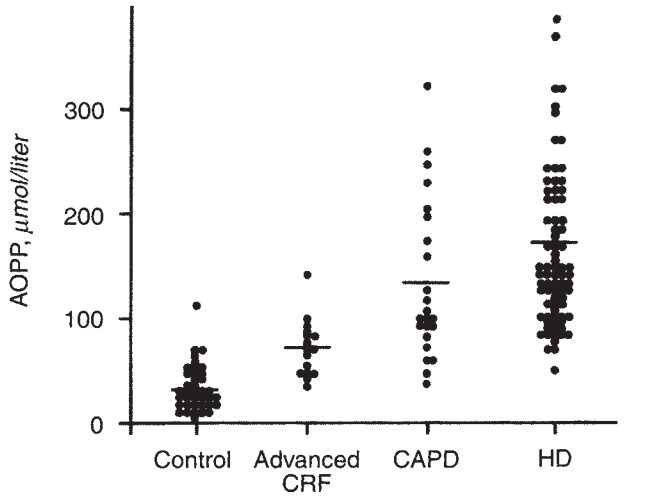

Fig. 8. *Plasma AOPP levels in undialyzed uremic patients, CAPD and HD patients as compared to controls.* Plasma AOPP levels were measured in uremic patients with advanced CRF, CAPD patients and HD patients, as compared to controls. Mean are indicated by horizontal lines. Differences between uremic patients and controls and between CAPD and HD patients are significant at $P < 0.001$.

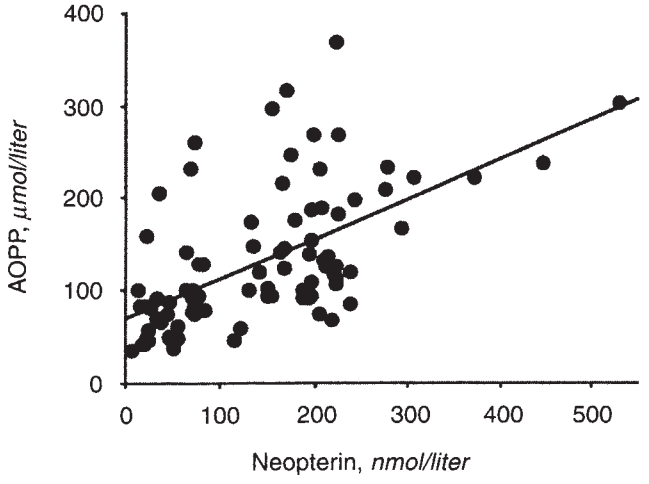

Fig. 9. *Relationship between AOPP and neopterin.* Regression analysis between plasma levels of AOPP and neopterin. Data are from 85 subjects including 17 patients with advanced CRF, 25 CAPD patients and 43 HD patients ($r = 0.59$; $P = 0.0001$).

Although the implication of oxidative stress in the pathophysiology of uremia has been suggested, little is known about the underlying mechanisms and a number of questions still remain to be solved. What is the respective influence of uremia and hemodialysis? What are the relationships with the chronic inflammatory state and the immune dysfunction associated with uremia? What are the relationships with clinical complications such as atherosclerosis and/or β_2-microglobulin amyloidosis? To date, evaluation of lipid peroxidation and of antioxidant systems has not provided reliable and sensitive tools to answer these questions.

Biochemical characterization of the species measured in our assay revealed that two distinct peaks at 670 and around 70 kDa account for the total AOPP level in plasma. Protein electrophoresis showed that the high molecular weight AOPP peak is mostly due to albumin that appears to form aggregates likely resulting from disulfide bridges and/or dityrosine cross linking. Likewise, the low molecular weight AOPP peak also contains albumin in its monomeric form. Interestingly, the molecular weight of the aggregates (670 kDa) is ten times that of monomeric albumin (67 kDa). Our AOPP assay does not react with non-oxidized native serum albumin, as evidenced by studies using purified HSA solutions or control plasma, or by plasma fractionation showing that the AOPP peak does not exactly match the albumin peak. On the other hand, we provide demonstration that oxidatively modified albumin leads to AOPP formation. Two interpretations are possible: either the low molecular weight-AOPP fraction may contain structurally and oxidatively-modified albumin that positives the AOPP assay; or alternatively, low molecular weight-AOPP are derived from other plasma proteins. Previous studies had shown that the numerous thiol groups of albumin account for the major antioxidant capacity of normal human plasma toward H_2O_2 or HOCl and that high concentrations of these oxidants are needed to form protein carbonyl [30]. This is in keeping with our

own data showing that for a solution of 60 mg/ml HSA or normal plasma, a minimum concentration of 5 mmol/liter HOCl is required to generate AOPP. Taking together, these data strongly suggest that AOPP measure highly oxidized proteins and especially albumin.

It is now well known that neutrophil-derived oxidants can alter the primary, secondary or tertiary structure of proteins to cause denaturation, increased hydrophobicity leading to protein fragmentation [40] and, at extremely high levels, protein cross linking. Such cross-linked products are less susceptible to proteolytic digestion and would be expected to form insoluble aggregates, as has been reported for many proteins including albumin [24]. The fact that AOPP levels positively correlate with dityrosine further supports the relevance of AOPP as an *in vivo* marker of oxidant-mediated protein damage [41]. With respect to the correlation between AOPP and the AGE-pentosidine, taken as a probe for the advanced Maillard reaction *in vivo*, the following comments can be made: increasing evidence links the Maillard reaction to oxidative pathway of injury to biological molecules both *in vivo* and *in vitro* as demonstrated by the inhibition of the formation of pentosidine in the absence of oxygen or in the presence of oxidant scavengers [34]. Conversely, glucose autooxidation proceeds with the release of free radicals and hydrogen peroxide thus potentiating the formation of AGEs [42]. Moreover, pentosidine levels correlate with the degree of renal failure and further increase on hemodialysis [43, 44].

The influence of lipids on AOPP determination is still not clear. *In vitro*, lipids are not necessary to form AOPP in a control plasma exposed to HOCl. In contrast, delipidation of plasma samples containing high concentrations of *in vivo*-formed AOPP halved AOPP level and plasma level of lipids or lipid peroxidation markers such as TBARS did not correlate with AOPP levels. The current explanation is that lipids are not necessary but may enhance the *in vivo* process of AOPP formation. Taking into account the fact that the oxidative modification of LDL is an important factor in the development of atherosclerosis [45], it is highly probable that AOPP and oxidized lipoproteins act in concert in this process.

Among the new analytical and biological approaches to investigate the complex mechanisms leading to uremia-related oxidative stress, electron spin resonance spectroscopy recently allowed to evidence the presence of oxidants in uremic plasma [46]. Oxidants were detected by their capacity to oxidize the spin trap 3,5-dibromo-4-nitrosobenzene sulphonate (DBNBS). Although these oxidants have yet to be isolated and precisely identified, available evidence indicates that they are dialyzable with an upper molecular weight limit of about 3 kDa, and they can be scavenged by high concentrations of ascorbic acid or glutathione [46]. The complexity of this technique, however, precludes its availability for clinical practice. In contrast, the methodology we propose, based on a spectrophotometric assay appears more simple and could easily be transferable from research to clinical laboratory investigation.

With respect to the possible pathophysiological significance of AOPP in the uremia-related immuno-inflammatory state, we found no relationship between AOPP levels and those of CRP as a nonspecific marker of inflammation. In contrast, plasma level of AOPP was positively correlated with that of neopterin which as we previously reported, closely reflects the monocyte activation state associated with chronic uremia [3]. Indeed, in this previous study we found that plasma levels of neopterin increased with the progression of chronic uremia in parallel with circulating levels of monocyte-derived pro-inflammatory cytokines or their specific inhibitors (including IL-1 and IL-1 receptor antagonist, TNF-α and its soluble receptors) as well as with those of T cell and B cell activation markers. The fact that the correlation between neopterin and monocyte-derived proinflammatory cytokines but not T or B cell activation markers persisted after adjustment of both parameters on creatinine clearance values suggests that neopterin accumulation is not solely explained by the loss of renal function and thus reflects the monocyte-macrophage activation related to uremia.

In conclusion, oxidant-induced protein damage appears to be an important component in the complex pathophysiology of the uremia-related immunoinflammatory disorder and could therefore take a part in uremic toxicity [47].

Acknowledgments

This work was supported by grants from the Extramural Grant Program, Baxter Corporation (MF and BDL) and by DK-45619 from the National Institute of Health (MF). We thank Françoise Tresset for skillful technical assistance, and Mathilde Labrunie, M.S. for statistical analysis.

Reprint requests to Béatrice Descamps-Latscha M.D., Ph.D., INSERM U25, Hôpital Necker, 161 rue de Sèvres, 75743 Paris CEDEX 15, France.

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