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Oxidatively-modified and glycated proteins as candidate pro-inflammatory toxins in uremia and dialysis patients

Review Article

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Summary. End stage renal disease (ESRD) patients accumulate blood hallmarks of protein glycation and oxidation. It is now well established that these protein damage products may represent a heterogeneous class of uremic toxins with pro-inflammatory and pro-oxidant properties. These toxins could be directly involved in the pathogenesis of the inflammatory syndrome and vascular complications, which are mainly sustained by the uremic state and bioincompatibility of dialysis therapy. A key underlying event in the toxicity of these proteinaceous solutes has been identified in scavenger receptor-dependent recognition and elimination by inflammatory and endothelial cells, which once activated generate further and even more pronounced protein injuries by a self-feeding mechanism based on inflammation and oxidative stress-derived events. This review examines the literature and provides original information on the techniques for investigating proteinaceous pro-inflammatory toxins. We have also evaluated therapeutic – either pharmacological or dialytic – strategies proposed to alleviate the accumulation of these toxins and to constrain the inflammatory and oxidative burden of ESRD.

Keywords: Protein damage – Oxidation – Glycation – AGEs – Inflammation – Reactive oxygen species – Nitric oxide – Uremia – Dialysis – Proteomics

Introduction: the inflammatory syndrome of ESRD – clinical and therapeutical aspects

End stage renal disease (ESRD) is often associated with a chronic inflammatory state, also described as micro-inflammation, characterized by a subclinical increase in cytokines, acute phase proteins, and oxidative stress indices (Ronco and Levin, 2002). Clinical and experimental evidence indicates that the higher the inflammatory insult, the higher the severity of morbidity and mortality in ESRD patients (Himmelfarb, 2005; Wagner et al., 2006). Endothelial dysfunction, atherosclerosis, cardiovascular calcification, malnutrition and muscle wasting, anemia, leukopenia, immune dysfunction, and dialysis-related amyloidosis are the main conditions associated with chronic inflammation and oxidative stress. However, the biological and clinical nature of this association is extremely complex and not completely understood. In consideration of the multi-factorial aetiology and extensive clinical consequences, we believe there is full reason to use the term ''inflammatory syndrome'' as the main definition to identify the inflammatory condition of ESRD.

Biochemical and clinical signs of inflammation and oxidative stress are particularly exacerbated in hemodialysis (HD) patients, appearing to a lower extent in patients on peritoneal dialysis and even more so in pre-dialysis patients (Simmons et al., 2005). This observation indicates a pro-inflammatory role for extracorporeal treatments due mainly to material bioincompatibility and dialysis fluid contamination with bacterial wall components. Other factors, often considered dialysis-independent, con-

tribute to sustain inflammation and include accumulation of pro-inflammatory uremic toxins, late responses of the immune system to acute effects of dialysis, recurrent infections, dyslipidemia, defective antioxidant protection, extensive use of pro-oxidant drugs such as iron and erythropoietin for the therapy of uremic anemia, and the release of hemoglobin-derived Fe-heme complex by microhemolysis during HD (Amore and Coppo, 2002; Ronco and Levin, 2002; Usberti et al., 2002b; Himmelfarb, 2005; Tsirpanlis et al., 2005).

In this paper we present an overview of the literature on serum protein damage in uremia and dialysis patients, describing the active role that these compounds may play in the genesis of ESRD-related inflammatory syndrome. Moreover, some preliminary aspects of investigation relative to the identification of proteinaceous uremic toxins and the characterization of dialysis membrane performance are described.

Leukocyte activation and inflammatory mediators in uremia and dialysis

The fact that inflammatory hallmarks progressively worsen with the evolution of renal damage with a zenith in HD patients, but are almost completely restored after renal transplantation (Simmons et al., 2005), clearly demonstrates the homeostatic role that the kidney plays in concert with other organs to constrain pro-inflammatory and pro-oxidant factors by clearance and/or metabolic processing. This indicates that uremia represents per se a pro-inflammatory state and at the same time suggests that dialysis treatment alone cannot surrogate the homeostatic role of the kidney. Paradoxically, there are dialysis-related factors that generate powerful pro-inflammatory and pro-oxidant events (Pupim et al., 2004). In HD these factors are essentially related to blood cell activation in the extracorporeal circulation (Fig. 1). Leukocytes play the most important role, although platelet activation has

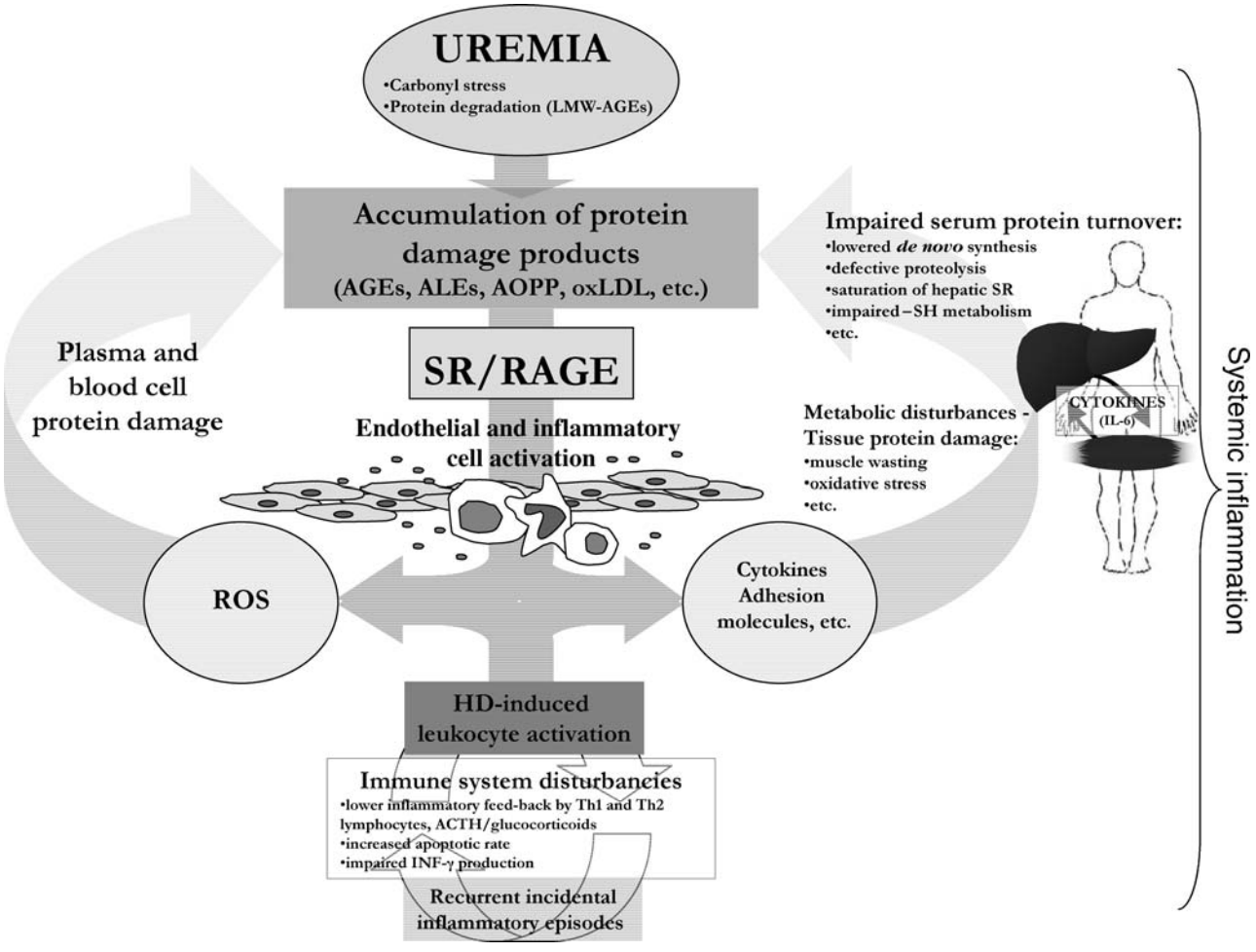

Fig. 1. The ''inflammatory loop'' sustained by scavenger receptor-dependent recognition of proteinaceous toxins that accumulate in uremic blood. Further details are reported in the text

been observed (Ballow et al., 2005) and chronic activation of endothelial cells has been associated to these events (Papayianni et al., 2002; Wautier and Schmidt, 2004).

The typical switching of lymphocyte subsets and monocytes to a pro-inflammatory phenotype observed in HD patients (Libetta et al., 2004) generates an abnormal production of proinflammatory/pro-oxidant cytokines (such as IL-1β and TNF-α as early monocyte products, INFγ, IL-6, IL-8 and others). In this context, there is overproduction and release of adhesion and chemotaxis factors (such as ICAM-1 and VCAM, selectins, MCP-1 and others) that may lead to a stable chronic condition in the long term (Horl et al., 2004), ultimately promoting leukocyte activation, adhesion, and migration into the sub-endothelial space as underlying events in atherosclerotic lesions and contributors to hemorrhagic and/or thrombotic manifestations (Musial et al., 2005; Tsirpanlis et al., 2005; Peng et al., 2006). The chronic stimulation of circulating mononuclear and polymorphonuclear leukocytes produces a state of energy and sustained apoptosis in these cell populations (Jaber et al., 2001; D'Intini et al., 2003; Galli et al., 2003a; Glorieux et al., 2003; Meier et al., 2002; Moser et al., 2003) and may lead to leukopenia and increased susceptibility to infections as main characteristics in the immune dysfunction of ESRD.

The balance between pro- and anti-inflammatory cytokines and the contribution of soluble cytokine receptors contribute to determine the complex series of events that characterize the complex inflammatory response observed in ESRD patients (reviewed in Guarnieri et al., 2005; Liangos et al., 2005; Macdougall and Cooper, 2005; Stenvinkel et al., 2005). Data suggest a key role for IL-6 and IL-6 gene polymorphisms in the biological consequences of this inflammatory syndrome, essentially increasing cardiovascular morbidity and mortality (Rao et al., 2005; Garibotto et al., 2006; Liu et al., 2006; Tuomisto et al., 2006). The major effects of this cytokine are produced in distinct sites far from its origin. IL-6 largely regulates the hepatic synthesis of the acute phase element C reactive protein (CRP) and down-regulates albumin synthesis. Elevated IL-6 levels are a primary stimulus for the production of the soluble intercellular adhesion molecule-1 (sICAM-1), which mediates leukocytes attachment to the endothelium surface and their subsequent migration. IL-6 may contribute to the development of atherosclerosis through various metabolic, endothelial, and pro-coagulation mechanisms. In this respect, both TNF-α and IL-6 inhibit lipoprotein lipase production in adipocyte cell lines, thus mediating lipolysis and dyslipidemia (Bologa et al., 1998).

An important role for inflammation and IL-6 levels in mediating malnutrition and muscle wasting in ESRD patients has been described. High levels of IL-6 have been associated with clinical signs of protein-caloric malnutrition such as hypoalbuminemia and cachexia. These signs are not the consequence of a nutritional defect, but rather reflect an extensive cannibalisation and altered metabolism of muscle protein stores. Garibotto et al. (2006) observed high IL-6 expression in the muscle of HD patients. This contributes to high circulating levels of the cytokine and a negative net protein balance in the muscle, possibly due to a consequential increased protein catabolic rate. Recent evidence suggests that loss of muscle mass in ESRD is attributable to activation of specific proteolytic mechanisms involving caspase-3 and the ubiquitin-proteasome system controlled by the insulin/insulinlike growth factor 1 and the phosphatidylinositol 3-kinase/Akt pathway (Mitch, 2006).

Nutritional aspects associated with inflammation can also contribute to negative muscle protein balance in ESRD, such as dietary restrictions and hyperleptinemia. Leptin, a protein-bound adipocyte-derived hormone, exerts sustained inhibitory effects on food intake while increasing energy expenditure. Serum levels are elevated in ESRD patients in correlation with inflammatory markers and muscle wasting (Mak et al., 2006). The leptin receptor belongs to the class I cytokine receptor superfamily and possesses strong homology to the signal-transducing subunits of IL-6 receptor. Experimental data on animal models of uremic cachexia associated with leptin receptor deficiency suggest a key role for this hormone in typical nutritional disturbances induced by nephrectomy (lowered weight gain, resting metabolic rate, efficiency of food consumption and changes of body mass and composition).

Inflammation, oxidative stress, and impaired NO metabolism

The first players of HD-related inflammation are PMN, or neutrophils, and oxidative stress appears to be one of the main biological consequences of the complement-dependent and independent-activation of these cells (Galli et al., 2005b; Himmelfarb, 2005).

Accordingly, chronic inflammation and oxidative stress in ESRD are characterized by increased hallmarks of biomolecule oxidation in serum, blood cells, and tissues. These include the lipid oxidation indices F_2-isoprostanes and oxidized LDL, the DNA oxidation index 8-OH-dG, and several protein oxidation and glycation markers

(Drueke et al., 2001; Ikizler et al., 2002; Lim et al., 2002; Muller et al., 2004; Li et al., 2006).

As a general definition, oxidative stress is the consequence of overproduction of reactive oxygen and oxygen/nitrogen species (ROS/RNS), such as H_2O_2, peroxynitrite ($ONOO^-$), superoxide ($O_2^{-\bullet}$), and the release/activation of pro-oxidant enzymes such as the heme protein myeloperoxidase (MPO) contained in PMN granules, transition metal decompartimentalization, and other factors. The enzyme MPO catalyses the H_2O_2 reaction of physiological concentrations of chloride anions (Cl^-) to give the potent oxidant HOCl (Hawkins et al., 2003). The latter, a reactive chloride species (RCS), is the most important pro-oxidant molecule produced by phagocytic cells and the main cause of tissue injury near the inflammatory foci.

Mononuclear leukocytes and tissue macrophages contribute to sustain oxidant stress events through the activity of the enzymes NADPH oxidase and inducible nitric oxide synthase (iNOS). Interleukins and anaphylatoxins produced during HD have been proposed to act as potent activators for NADPH oxidase (Morena et al., 2005).

Among its multiple physiological roles associated with arterial wall relaxation, platelet function, neurotransmission, intra- and inter-cellular signalling, etc., nitric oxide ($NO^\bullet$) is a well recognized mediator of inflammation (Modlinger et al., 2004), and RNS may result potentially dangerous when the extent of their production and reactivity increase in association with inflammation and oxidative stress events. Activated macrophages generate large $NO^\bullet$ fluxes via up-regulation of iNOS while simultaneously producing superoxide. Individually, both $NO^\bullet$ and $O_2^{-\bullet}$ are free radicals, and though neither is highly reactive alone, radical–radical combinations are highly reactive (Butterfield, 2006). These two radicals react with each other to provide the more reactive species peroxynitrite which, in the presence of CO_2, is responsible for hydroxylation and nitration of protein in vivo (Alvarez and Radi, 2003). As a consequence, peroxynitrite is recognized as a toxic $NO^\bullet$ derivative with possible detrimental effects on endothelial function (Zou et al., 2004). Nitrating agents can also be produced in vivo from other reactions such as nitrite oxidation by peroxidase activity.

Changes in physiological function and metabolism of $NO^\bullet$ have been reported in ESRD-related inflammation and may contribute to the pathogenesis of conditions such as endothelial dysfunction, hypertension, atherothrombosis and malnutrition (reviewed in Modlinger et al., 2004; Brunini et al., 2006). Inflammation/oxidative stress is responsible for increased systemic production of $NO^\bullet$ observed in uremia and for impaired synthesis, molecular recognition, and processing of $NO^\bullet$ in the endothelial wall (Vaziri, 2001; Cross, 2002). Protein damage in the sub-endothelium by glycoxidation reactions is one of the molecular mechanisms that can hamper $NO^\bullet$ flux from the endothelium toward smooth muscle cells, ultimately reducing vessel elasticity. As a consequence, this is recognized as a direct pathogenic event in the cardiovascular disease of uremia and diabetes. Paradoxically, impaired $NO^\bullet$ metabolism and biological functions may contribute to increased susceptibility to oxidative stress and protein damage in uremic tissues (Asahi et al., 2000). $NO^\bullet$ disturbances in ESRD have been reported to increase platelet activation, adhesion, and vessel rigidity and may be related to a decreased availability of the $NO^\bullet$ synthesis precursor L-arginine – possibly due to reduced dietary intake or defective amino acid metabolism – and blood accumulation of endogenous L-arginine analogues which act as NOS inhibitors (Conte et al., 2005).

Hallmarks of protein damage in uremia and dialysis patients

Protein modifications by ROS/RNS, reactive alkenals, or other glycation or glycoxidation agents lead to hydroxylation of aromatic groups and aliphatic amino acid side chains, nitration of aromatic amino acid residues, nitrosylation of sulfhydryl groups, sulfoxidation of methionine residues, chlorination of aromatic groups and primary amino groups, cross-linking to conversion of amino acid residues to carbonyl derivatives (reviewed in Butterfield and Stadtman, 1997; Stadtman and Levine, 2003; Uchida, 2003). These changes result in different types of structural damage, functional alterations, and modified protein turnover by proteolysis systems. Both the different indices of free amino acid and protein modifications have been found to accumulate in the uremic blood and might have a role as pro-inflammatory and pro-oxidant uremic toxins (see below).

In ESRD the most common hallmarks of protein damage reported in the literature and discussed in this review are tyrosine-derived products, protein carbonyls, and advanced glyc(oxid)ation end products (AGEs). Examples of these indices are shown in Fig. 2A–C.

Tyrosine modifications and advanced oxidation protein products (AOPP)

Detection of tyrosine modifications currently represents one of the most sensitive and specific tools to detect end products of specific oxidation/nitration pathways. In particular, the modification of tyrosine residues leads to the

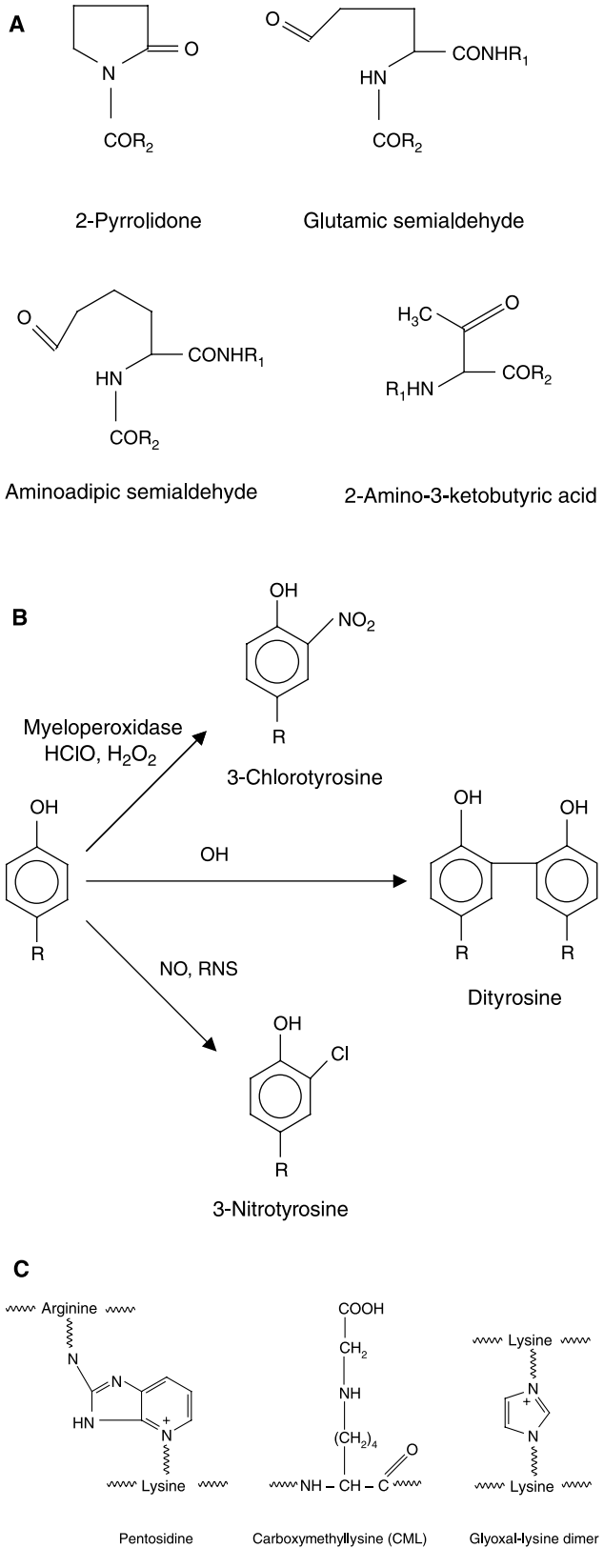

Fig. 2. Some protein damage hallmarks among the most frequently assessed in human plasma: protein oxidation products (**A**), tyrosine-derived indices (**B**) and glycation end-products or AGEs (**C**)

3-chlorotyrosine, 3-nitrotyrosine, or dityrosine depending on the predominant species involved in the reaction. Plasma proteins in HD patients contain elevated levels of 3-chlorotyrosine (Himmelfarb et al., 2001). Since this hallmark is a specific product of MPO-catalyzed reaction, this suggests a precise and important role for PMN-initiated oxidative reactions as a primary cause of oxidative stress and protein damage in uremia.

Tyrosine nitration to form 3-nitrotyrosine has been investigated in ESRD, and it has been found to increase from 2 to 10 fold in these patients compared to healthy individuals, suggesting a high flux of RNS and/or defective scavenging systems (Mitrogianni et al., 2004).

Witko-Sarsat and colleagues isolated and provided biochemical and immunological characterization of dityrosine-containing proteins in the plasma of patients on regular dialysis (Witko-Sarsat et al., 1996; Descamps-Latscha et al., 2005). These cross-linking products were designated as AOPP. Since AOPP plasma levels were found to closely correlate with creatinine clearance, they were proposed as markers of the progression of chronic renal failure and a proof of the correlation existing between uremia and oxidative stress. This association was also supported by the fact that AOPP correlates with neopterin, a marker of macrophage activation. In addition, HOCl-treated albumin (an in vitro source of AOPP) and in vivo-generated AOPP triggered neutrophil and monocyte oxidative burst, thereby acting as true inflammatory and oxidative stress mediators (Witko-Sarsat et al., 2003a). Collectively, these studies on modified proteins and amino acids as biomarkers of oxidative stress strongly suggest that MPO-derived HOCl could represent one of the most important pro-oxidants in HD patients.

Protein carbonyls

Oxidative modifications of proteins are induced directly by ROS/RNS or through the reaction with oxidative stress by-products. Cysteine and methionine are particularly prone to oxidative attack by ROS/RNS. Protein modification by direct oxidative attack on Lys, Arg, or Thr or by secondary reaction of Cys, His, or Lys residues with reactive carbonyl compounds leads to the formation of a series of protein carbonyl derivates (Butterfield and Stadtman, 1997; Stadtman and Levine, 2003). Protein carbonyls can be generated also through oxidative cleavage of proteins, via the α-amidation pathway or through oxidation of glutamine side chains, leading to the formation of a peptide in which the N-terminal is blocked by an α-ketoacyl derivative.

The introduction of carbonyl groups into proteins occurs by Michael addition reactions of α,β-unsaturated aldehydes, such as 4-hydroxy-2-nonenal, malondialdehyde and 2-propanal, with either the amino group of lysine, the imidazolone moiety of histidine, or the sulfhydryl group of cysteine (Butterfield et al., 2002). These adducts are identified also as advanced lipoxidation end-products (ALEs) (Thorpe and Baynes, 2003). Carbonyl groups are introduced into proteins also by addition of reactive carbonyl derivatives (ketoamines, ketoaldehydes, deoxysones), produced by the reaction of reducing sugars or their oxidation products, and in the amino group of amino acid residues by mechanisms such as glycation and glycoxidation that eventually form AGEs (Langer et al., 2006).

Several studies have demonstrated that plasma reactive carbonyl compounds (measured as hydrazone formation after reaction with 2,4-dinitrophenylhydrazine) are increased ten-fold in ESRD patients (Galli et al., 2005a; Miyata et al., 2001).

Advanced glyc(oxid)ation end-products (AGEs)

This class of compounds has been investigated extensively and their analysis appears to be a robust tool to assess the extent of protein damage in vivo by carbonyl stress and glycoxidation reactions in uremia and dialysis patients.

Glycation is one of the many non-enzymatic protein modifications that contributes to protein aging, but it may be important also in physiological responses and pathological processes. Advanced protein glycation mechanisms and products have been reviewed by Thorpe and Baynes (2003) in a previous number of this journal. In this reaction, initially investigated by food chemists and identified to proceed through a series of steps known as Maillard (or browning) reaction, a large number of intermediates and AGE structures are formed by enolization, dehydration, cyclization, fragmentation, and oxidation reactions. In the classical protein glycoxidation mechanism described in the Maillard reaction, an aldehyde group of a reducing sugar undergoes a nonenzymatic condensation reaction with the amino group of a protein residue to form a Schiff base adduct. The latter rearranges to form a ketoamine (fructosamine) adduct, which is described with the name of Amadori product. Both the Schiff base and ketoamine adducts are stabilized in cyclic furanose and pyranose configurations. The Amadori adduct formed from glucose in vivo undergoes non-oxidative rearrangement and hydrolysis reaction, forming 1- and 3-deoxyglucosones (1DG, 3DG) preserving the carbon skeleton of the sugar precursor. The Schiff base and Amadori adduct are prone to oxidation, especially in the presence of transition metal ions, and fragment to short chain sugars and reactive intermediates, such as glyoxal (GO) and methylglyoxal (MGO). Glucosone (GLO) and GO are intermediates in the formation of N^{ε}-(Carboxymethyl)lysine (CML) and are produced from Amadori product and also from peroxynitrite-mediated oxidation of glucose. These reactive dicarbonyl compounds, described as intermediates formed during the second stage of Maillard chemistry, react with lysine and arginine residues of proteins to produce a wide range of AGEs and cross-links during the third and final stage of the classical reaction pathway.

The imidazolium salt cross-link, gliossal-lysine-dimer (GOLD), is included in the glyoxal-derived AGE structure. Also methylglioxaldiimine, a MGO-protein adduct, is produced by cleavage of the Amadori product in the Maillard reaction. Methylglyoxalediimine may be converted to methylglyoxal-derived AGE structures, such as N^{ε}-(Carboxyethyl)lysine (CEL), argypyrimidine, and imidazolone, and to imidazolium salt cross-links, such as the methylglyoxal-lysine dimer (MOLD). AGEs such as fluorescent vesperlysine and cross-links retain the intact carbon structure of glucose and appear to derive directly from glucose. In contrast, glucose-derived pentosidine requires oxidative cleavage and loss of one carbon atom. Other AGEs, such as CML and CEL, require oxidative fragmentation of the glucose carbon skeleton. Several Maillard intermediates and AGEs have been detected in ESRD (Wada et al., 1999; Floridi et al., 2002; Galli et al., 2005a; Thornalley, 2006).

The role of free reactive carbonyls and oxidative stress in the genesis of AGEs and ALEs has been unquestionably demonstrated. Reactive carbonyls derive from polyunsaturated fatty acids, sugars, glycolytic intermediates, ascorbic acid, and free amino acids through oxidative stress and co-factors such as transition metals. Anderson and colleagues showed that activated neutrophils produce α-hydroxy and α,β-unsaturated aldehydes from hydroxyl amino acids in high yield through activity of the MPO-H_2O_2-chloride system (Anderson and Heinecke, 2003). Activated neutrophils convert L-serine to glycoaldeyde and α-hydroxyaldehyde that mediate protein cross-linking and CML formation. L-threonine oxidizes similarly to 2-hydroxypropanal and its dehydration product, acrolein. This latter molecule is an extremely reactive α,β-unsaturated aldehyde that can alkylate proteins and nucleic acids (Pocernich et al., 2001). Aldehyde generation requires neutrophil activation and the free hydroxyl moiety of an amino acid. The generation of gly-

coaldehyde, 2-hydroxypropanal, and acrolein by activated phagocytes may thus play a role in AGE formation and tissue damage during inflammation (Himmelfarb et al., 2001).

Analysis and clinical interpretation of AGE accumulation

Glycation reactions and oxidative stress contribute to increased cardiovascular risk of ESRD patients, but may contribute also to the genesis and progression of diseases such as uremic anemia (Usberti et al., 2002a) and amyloidosis (Niwa, 2001). As a consequence, there is growing clinical interest in the evaluation of AGE formation and accumulation in ESRD.

Fluorescent AGEs are often used as in vivo hallmarks of metabolic stress by the Maillard reaction. However, this reaction also produces non-fluorescing products, such as MOLD, GOLD, the glyoxal-Lys dimmer, which reach blood concentrations in uremia and diabetes patients several-fold higher than fluorescing epitopes such as pentosidine. Some of the most important AGE hallmarks and their biological precursors are reported in Table 1.

It should be noted that AGEs markedly accumulate either as free or protein-bound forms in uremic serum independently from the presence of diabetes (Langer et al., 2006). However, hyperglycaemia is a frequent event in ESRD and further exacerbates AGE accumulation. Dietary intake and intestinal absorption can contribute to sustain AGE levels in these patients (Ahmed et al., 2005).

In vivo proteolytic processing of AGEs produces a heterogeneous class of circulating by-products described as free glycation adducts (Agalou et al., 2005b) or low molecular mass (LMM)-AGEs (Floridi et al., 2002) that maintain biological properties similar to the modified proteins from which they originate. Together with free carbonyls and pro-oxidant species, they can contribute to glycation reactions and protein damage in tissues. Importantly, the reduced molecular mass of proteolysis products, compared to precursors, facilitates removal during dialysis.

Table 1. Some AGEs and their most relevant biological precursors. Further details on these protein damage products and their formation are reported in the text

AGEs	Precursor(s)
Pentosidine	Carbohydrates
Carboxymethyllysine	Carbohydrates, lipids, amino acids
Imidazolone	Carbohydrates
Glyoxal-lysine dimer	Carbohydrates, lipids, amino acids
Methylglyoxale-lysine dimer	Carbohydrates, lipids, amino acids

Biological sample analysis and mass transfer quantification during dialysis are key aspects for investigating the biochemical and clinical implications of AGE accumulation in ESRD. Exact identification and quantification of AGEs in biological fluids can be accomplished by laboratory analysis based on HPLC and GC techniques coupled with spectroscopy (McCance et al., 1989; Sell et al., 1991; Floridi et al., 1999, 2002) or mass detection systems (reviewed in Thornalley, 2005; Niwa, 2006). The analysis of fluorescing epitopes has been largely adopted in clinical studies, furosine and pentosidine being the most investigated. The fact that their levels are much lower than other non-fluorescing AGEs (such as MOLD, GOLD, or CML) is not a realistic problem, since method sensitivity and blood levels of these hallmarks make analysis reliable and easily applicable. Clinical investigation of AGE levels in uremic serum has been performed using direct spectrofluorimetric analysis of fluorescing epitopes (without chromatographic separation of test molecules). In this case, analyses may require considerable validation to avoid possible false results. Immunodetection methods have been developed to trace epitopes such as CML and pentosidine (Taneda and Monnier, 1994) or imidazolone that has been detected in the aorta of uremic subjects (Takayama et al., 1998). More recently, the skin autofluorescence assay was proposed as a non-invasive test to quantify tissue AGEs, which was validated against skin biopsies. In accordance with the view that tissue AGEs may represent a measure of cumulative metabolic stress and pro-inflammatory status in uremia, it has been observed that skin autofluorescence is a strong, independent predictor of overall and cardiovascular mortality in ESRD (Meerwaldt et al., 2005).

Isolation of large solutes from uremic blood can be achieved by different techniques (as ultrafiltration or chromatographic separation) and could be an important step toward their molecular characterization and analysis. Electrophoresis carried out either under denaturating or non-denaturating conditions is a basic technique of great help to isolate large solutes from body fluids and tissue homogenates. When coupled with immunological detection by western blotting or mass analysis, it can provide also a tool for the identification and quantitative analysis of individual proteins or specific epitopes related with physiological post-translational modifications and protein damage products (see below).

The adsorption of AGEs by sorbent resins (BetaSorb^{TM}) is an example of chromatography that has been used to characterize the pro-inflammatory power of uremic serum samples (Morena et al., 2003). The in vitro re-

moval of fluorescent AGE epitopes such as pentosidine, in fact, significantly reduces the pro-inflammatory activity of the uremic serum or plasma. Some of us (Galli F, unpublished observation) observed that the recirculation of uremic plasma into mini-module cartridges containing the same sorbent resin can decrease from 27 to 45% in the different LMM-AGEs (MW <5000 Da) showing the fluorescence of pentosidine. Moreover, the fact that these sorbents can remove other molecules (peptides and small proteins such as β2-microglobulin, leptin, complement factor D, angiogenin and a number of cytokines) suggests future clinical application in dialysis and hemoperfusion techniques applied to acute or chronic inflammatory conditions (Winchester et al., 2004).

Because of its biochemical features and relative abundance, serum albumin is the preferential (sacrificial) target of glycation and oxidation reactions in the circulation (Himmelfarb and McMonagle, 2001; Mera et al., 2005). Actually, this protein is highly susceptible to metal-catalysed oxidation (Wratten et al., 2001; Anraku et al., 2004), but at the same time it behaves as an important antioxidant in that it contains one highly reactive sulfhydryl group at position 34 (Cys-34) preferentially present in the reduced state. This thiol group reacts readily with oxidants such as H_2O_2, $NO^{\bullet}$ and HClO, and it is responsible of the thiol buffering activity of albumin towards small mass thiols such as the free amino acids Hcy and Cys to form mixed disulfides. Due to these characteristics, the analysis of human serum albumin reveals a mixture of mercaptalbumin (MA; reduced form) and nonmercaptalbumin (NA; oxidized form). MA contains Cys-34 in the reduced form, while other serum proteins contain little or none. NA comprises at least two groups of molecules. The major NA component is a mixed disulfide with cysteine or glutathione (NA-1). The other is a more highly oxidized product than the mixed disulfide, in which the thiol group has been oxidized to the sulfenic (SOH), sulfinic (SO_2H), and sulfonic (SO_3H) states (NA-2), the proportions of which are extremely small in extracellular fluids.

The overall blood thiol redox status is impaired in uremia and dialysis patients (Himmelfarb et al., 2002; Dursun et al., 2005; Lucchi et al., 2005) and this may contribute to increase albumin damage due to carbonyl and oxidative stress. In fact, increased levels of carbonyl compounds are correlated with the oxidation of albumin in uremic patients.

Pentosidine fluorescence in uremic plasma is mainly associated to albumin (Galli et al., 2005a), which also contains a large portion of total protein carbonyls (Himmelfarb and McMonagle, 2001). However, MALDI-MS analysis of fatty acid-free albumin from uremic individuals has shown minimal modifications of this protein that might be consistent with AGE formation through reaction with α-oxoaldheydes (molecular shift of +498 Da in ESRD and +438 Da in ESRD on HD) (Thornalley et al., 2000).

Cell responses to oxidative and glycoxidative damage: the "inflammatory switch"

The scavenger receptor (SR) system

There is general consensus on the fact that the accumulation of glycoxidation products, such as pentosidine, CML, and imidazolone in specific tissues and circulating proteins, represents a pathogenic event of clinical relevance in cardiovascular disease and other conditions associated with aging (reviewed in Simm et al., 2004; Wautier and Schmidt, 2004). The interaction between cells and AGE proteins triggers biological responses, including endocytic uptake and degradation of AGE proteins (i.e. in the liver by sinusoidal cells such as Kupffer and endothelial cells), metabolic activation and secretion of inflammatory cytokines by phagocytes and endothelial cells, stimulation of monocyte chemotaxis, proliferation of vascular smooth muscle cells, quenching of $NO^{\bullet}$ activity with impaired smooth muscle relaxation in the arterial wall, collagenase secretion in sinovial cells and osteoclast-induced bone resorption. These responses are mediated by a family of receptors that include the AGE receptor (or RAGE) belonging to the immunoglobulin superfamily and originally purified from bovine lung endothelial cells, the galectin-3/80 K-H/oligosaccharyltransferase-48 (OST-48) complex and other receptors still under investigation (Horiuchi et al., 2003). Scavenging activity against AGE proteins was identified also for receptors such as CD36 and for other members originally recognized to specifically mediate the endocytosis and metabolism of modified and native lipoproteins such as the macrophage types I and II class A scavenger receptors (MSR-A), SR-BI and lectin-like ox-LDL receptor 1 (LOX-1) (Horiuchi et al., 2003).

Scavenger cells are widely represented in human tissues mostly in association with vascular components and, also in consideration of the pleiotropic nature of SR function, they form a robust and integrated system of control and defence in the whole organism. The constant recognition and removal by scavenger cells of oxidized and glycated proteins by the different members of the SR system is part of this advanced homeostatic response intimately connected with inflammation, foreign and cancer cell killing, tissue

remodelling and repair during normal growth or degenerative processes. Inflammatory cells and several of the cell components in the vessels (such as PMN, monocyte-machrophages, endothelial cells and smooth muscle cells) can express to different extents highly inducible forms of SR and this represents a key biological aspects to explain how AGE accumulation might sustain inflammation and endothelial dysfunction. Once activated the SR response stimulates the metabolism of SR-bearing cells, which can produce large amounts of ROS/RNS (Witko-Sarsat et al., 2003b) and the release of mediators such as pro-inflammatory cytokines as TNF-α IL-6, TGF-β, and arachidonic acid-derived bioactive lipids (Simm et al., 2004). Each category of these mediators is important in the coordination of tissue and vasculature functions with inflammatory events. On the other hand, these mediators can spread inflammation and oxidative stress at systemic level.

Cell stress response pathways: heat shock proteins and thioredoxin

Oxidative and glycoxidative challenges belong to a series of adverse events of exogenous and/or endogenous origin. To cope with stress, eukaryotic cells have evolved specific response networks (cellular stress response). These responses detect and control different forms of stress and include heat shock response (Poon et al., 2004; Calabrese et al., 2006; Mancuso et al., 2007) and the thioredoxin system (Nakamura, 2005). Heat shock proteins (Hsps) form a highly conserved system responsible for the preservation and repair of correct protein conformation. Recent studies have shown that heat shock response establishes a cytoprotective state in a wide variety of human diseases, including inflammation, cancer, aging, and neurodegenerative disorders (Poon et al., 2004; Calabrese et al., 2006). Given the broad cytoprotective properties of heat shock response, there is a great interest in discovering and developing pharmacological agents capable of inducing heat shock response. The thioredoxin system (Trx, Trx reductase, peroxiredoxins, and NADPH) is a ubiquitous thiol oxidoreductase system that participates in the reduction/oxidation (redox) status at cellular level (Nakamura, 2005). Together, Hsps and components of the thioredoxin system form a powerful approach involved in many central intracellular and extracellular processes, including cell proliferation, redox regulation of gene expression and signal transduction, protection against oxidative stress, anti-apoptotic functions, growth factor and co-cytokine effects, and regulation of the extracellular redox state.

It has been demonstrated recently that Hsps (Hsp27) can be structurally modified by MGO in endothelial and glomerular mesangial cells (Padival et al., 2003; Schalkwijk et al., 2006). This modification could be a possible mechanism by which glycoxidation products decrease cell ability to react against oxidative conditions. The importance of Hsps to counteract AGE toxicity has been supported by evidence of Hsp70 over-expression in a macrophage cell line acutely exposed to glycated serum (Bassi et al., 2005). After longer exposure Hsp70 levels decreased, possibly because of direct Hsp70 involvement in refolding damaged proteins (Bassi et al., 2005). Interestingly, heme oxygenase-1, also referred to as Hsp32, an important enzyme involved in antioxidant cell defense because of its ability to degrade pro-oxidant heme and generate antioxidant molecules such as bilirubin, was over-expressed in the blood of young uremic patients under short-term HD, whereas its levels were reduced in long-term HD (Maroti et al., 2004). Taken together, these findings raise the possibility that Hsps over-expression is a common mechanism by which cells attempt to neutralize the pro-oxidant insult of ESRD/HD. However, this cytoprotective effect does not produce effective long-lasting self-protection since it tends to be lost in the case of a prolonged oxidant attack.

Protein damage products as candidate pro-inflammatory uremic toxins

The concept of "uremic toxin"

It is well known that uremic blood contains different leukocyte-activating pro-inflammatory components, but none of them have been unquestionably characterized and quantified (Schwedler et al., 2001; Agalou et al., 2005a; Vanholder et al., 2006). Available information recently monitored by the European Uremic Toxin (EUTox) Work Group (Vanholder et al., 2003), allowed to identify only 90 compounds that reside in the definition of ''uremic toxin''. The examination of biochemical and clinical aspects concerning toxin accumulation in ESRD clearly shown characteristics of extreme heterogeneity concerning chemical and physical properties. So far identified uremic toxins show a wide molecular weight range (from small to large solutes), different blood concentrations and variable characteristics of hydrophobicity. These aspects influence the efficacy of dialysis strategies in removing solutes (Yavuz et al., 2005; Vanholder et al., 2006). Regarding molecular weight, 68 compounds are smaller than 500 Da (small molecules), 10 are between 500 and 12,000 Da (middle) and 12 exceed 12,000 Da (large). Twenty-five

solutes (28%) are protein-bound and mainly small molecules (except for leptin and retinol-binding protein).

However, in the systematics of uremic toxins, the term ''protein-bound'' can generate confusion. In this respect, it is advisable to identify the type of binding distinguishing between covalently bound small solutes and irreversible (end-products) or reversible protein modifications. For instance, Hcy has to be included in the category of reversibly protein-bound small solutes. In fact, Hcy forms a reversible disulfide bond with the Cys-34 residue of albumin, the most abundant free thiol in plasma (Perna et al., 2003). On the contrary, pentosidine similar to other AGEs, became an integrated part of the protein structure in which Arg and Lys (as well as other nucleophilic groups in proteins such as the side chains of His, Ser, Thr, Trp and Tyr) are stably and irreversibly modified by glyc(oxid)ation to generate a intra- or inter-molecular cross-link (Thorpe and Baynes, 2003). Thus, in this case there is no binding, but an irreversible structural change with the formation of an end-product different from original substrates (reducing sugar and glucose products, short chain sugars, reactive carbonyls and several other glycation and lipoxidation intermediates). The same can be applied to the case of oxidative stress generated end-products such as di-Tyr (Giulivi et al., 2003) or Lys and Hys oxidation products (Uchida, 2003) in which again there is no binding but rather an irreversible molecular change. Thus, we suggest to abolish the misused general term ''protein-bound'' that for systematic purposes should be limited to reversibly bound small solutes. In the other cases, it should be substituted by a correct identification of individual end-product categories (for instance: mixed disulfides, AGEs, ALEs, AOPP, etc.), together with the reaction species and pathway(s) involved in their formation (for instance: AGE-pentosidine, AGE-CML, ALE-MDA). When appropriate, the name of individual amino acid residues or protein involved should be specified (for instance: MDA-Lys or MDA-albumin, 4-HNE-albumin, etc).

Proteinaceous (large) toxins

The hypothesis that large proteinaceous solutes with pro-inflammatory activity accumulate in uremic serum has been proposed in the last years. It originated from in vitro evidence (Morena et al., 2003) and the clinical observation that inflammation persists even after ''adequate dialysis therapy''. In fact, it is intriguing to observe that conventional hemodialysis techniques (cut-off limit of dialysis filters of approx. $\leq$30 kDa) carried out with synthetic (highly biocompatible) dialysis membranes and ultrapure dialysate can not satisfactorily correct biochemical or clinical signs of inflammation in ESRD patients. Dialysis carried out with standard low and high flux dialysers removes efficiently but almost exclusively small/middle molecules ($\ll$5000 Da) from uremic blood (i.e. the ''classical uremic toxins'') and only small amounts of larger solutes such as β2-microglobuline (MW = 11,000 Da). These latter molecules, once modified by glycation, show a further lowering of mass transfer through either neutral or charged membranes (Randoux et al., 2001). Protein modifications can affect solute filterability by changing both solute MW and net charge, which may further contribute to increased physical hindrance of solutes to pass through pores of charged dialysis membranes. Accordingly, blood levels of AGEs and their proteolysis by-products, as well as ROS/RNS-derived protein damage products, are not efficiently corrected by conventional dialysis methods (Schwenger et al., 2001; Floridi et al., 2002; Galli et al., 2005b; Thornalley, 2006). Also small solutes with pro-inflammatory activity are not efficiently cleared because of their physical and chemical behaviour in uremic blood, and this can contribute to increase large solute formation. Urate may be present as mononuclear cell-activating crystals (Bordoni et al., 2005) and p-cresol, which other than being ''protein-bound'', has been reported to enhance oxidation susceptibility of serum proteins (Wratten et al., 2001).

These aspects may lead to the simple conclusion that several classes of high molecular weight solutes (HMWs or large solutes) accumulate in uremic serum and support the hypothesis of a role as pro-inflammatory toxins. These comprise a heterogeneous group of proteinaceous molecules of various origin. Some are physiological solutes which accumulate in uremic blood through the combination of an abnormal metabolism of uremic tissues and the pro-inflammatory effect of HD. These include complement-derived components, hormone-related proteins and peptides, ligands and soluble receptors, IgG light chains, and PMN granule components (Cohen et al., 2001; Deppisch et al., 2001; Cohen, 2003; Glorieux et al., 2003; Mak et al., 2006).

Other HMWs are the result of oxidative and glycation processes directed toward biological fluid and tissue proteins (McCance et al., 1989; Monnier et al., 1992; Lamb et al., 1993; Odani et al., 1996; Miyata et al., 1998; Descamps-Latscha et al., 2005). In fact ESRD, similar to diabetes, is a condition that sustain glycation and oxidative stress-related protein damage, and this is considered to represent a main underlying event of accelerated aging (Niwa, 1999; Himmelfarb et al., 2001; van Ypersele de Strihou, 2003; Jimenez et al., 2005). The broad molecular alterations occurring in the blood of uremia and

dialysis patients are sustained by the concerted action of oxidative stress and massive accumulation of free reactive carbonyls, a condition described as ''carbonyl stress'' (Miyata et al., 2001). As discussed above, these carbonyl moieties derive from polyunsaturated fatty acids, sugars and glycolytic intermediates, ascorbic acid, and free amino acids through the contribution of oxidative stress co-factors such as transition metals and MPO release. Therefore, the HD-dependent activation of PMN and the activity of the MPO-H_2O_2-chloride system play a central role in this context producing for instance reactive aldehydes such as glycoaldehyde, 2-hydroxypropanal, and acrolein (Anderson and Heinecke, 2003).

Scavenger receptor (SR)-dependent recognition of modified proteins: the ''inflammatory loop'' of ESRD

A key aspect to understand the role of modified proteins, and in general of uremic HMWs, in the pathogenesis of ESRD-related inflammation is that they may be at the same time products and triggers of inflammation and oxidative stress. This dual role is at the origin of a self-feeding loop (Fig. 1). The loop is basically the consequence of SR-dependent recognition of HMWs by inflammatory (scavenger) cells, i.e. PMN, monocyte-macrophages, and endothelial cells (Simm et al., 2004; Wautier and Schmidt, 2004). This recognition is addressed to the elimination (cleaning) of modified proteins from the circulation and tissues. When HMW production by protein damage in biological fluids and tissues progressively increases as a consequence of the onset and progression of kidney failure, HMWs increasingly challenge the SR system in the whole body. Therefore, in uremia and dialysis, the homeostatic role of SR responses paradoxically can shift from physiological response of defence and ''molecular cleaning'' to cause of inflammation. This abnormal chemistry, thus, produces a continuous stimulation of the respiratory burst in SR-bearing cells (Witko-Sarsat et al., 2003b), which ultimately leads to a sustained oxidative burden with the neoformation of further HMWs from serum and tissue proteins.

Direct confirmation of this model based on SR-recognition of modified proteins came from in vitro evidence that selective removal with sorbent resins of pentosidine-like fluorescing HMWs, markedly decreased pro-inflammatory properties of uremic serum, as assessed by TNF-α production in U937 monocyte-machrophages (Morena et al., 2003), and importantly, the pro-inflammatory activity of the solutes removed was demonstrated to be largely RAGE-dependent. HD treatment, particularly the use of poorly biocompatible hemodialysers, was found to enhance expression of members of this class of SR in circulating mononuclear leucocytes (Friedlander et al., 1996; Chmielewski et al., 2005; Wu et al., 2005). CD36, which mainly scavenges oxidized LDL particles, could contribute to this RAGE-dependent effect due to functional overlap between SR families expressed by inflammatory cells, liver sinusoidal cells, and Kupffer cells (Horiuchi et al., 2003).

The continuous SR-dependent activation of inflammatory and vascular cells provokes the chronic release of mediators such as TGF-β and pro-inflammatory cytokines such as TNF-α and IL-6 (Simm et al., 2004). Increased levels of mediators in the circulation are responsible for pleiotropic secondary effects on peripheral (non-inflammatory) tissues that cause systemic inflammation and extend oxidative stress. This sequence of events can evolve to chronic-degenerative processes and specific disease states as proposed in the case of endothelial lesions and atherosclerotic plaque formation. Accordingly, RAGE expression in the arteries of non-diabetic uremic subjects has been suggested to affect the increased atherosclerotic susceptibility of these patients (Greten et al., 1996).

Consistent with the pleiotropic effects and self-feeding nature of this inflammatory syndrome, HMWs can generate also without the involvement of oxidative or glycation events and tissue SR mediation. This is the case, for instance, of the production of acute phase proteins in the liver or complement activation products and abnormal release of PMN granule content. Again, the activation of resident inflammatory cells produces local generation of HMWs in tissues that can be released in the circulation intact or after partial proteolytic degradation.

Other factors contribute to the progression of systemic inflammation in uremia and dialysis (Fig. 1). Among them, the dysregulation of the negative/anti-inflammatory feedback exerted by ACTH, glucocorticoids, and cytokines such as IL-4, IL-10, and TGF-α is one of the most important. This is part of the consequences produced by the prolonged and sustained activation of the inflammatory response by dialysis bioincompatibility and uremic intoxication. In this respect, the entire immune (cellular and humoral) response can be compromised, and leucopoenia and immune cell energy are main symptoms of this immune dysfunction.

Strategies to correct the accumulation of pro-inflammatory uremic toxins

Inflammation in ESRD is still a condition that can not be cured with dialysis and pharmacological therapies. Future

research steps should be oriented to identify, and hopefully prevent, as earlier as possible the onset of the inflammatory loop sustained by the accumulation of HMWs. Hallmarks of this loop should be evaluated together with carbonyl and oxidative stress hallmarks already in pre-dialysis and if possible in the early phases after the diagnosis of kidney failure, since they may provide important information about the clinical evolution of the inflammatory syndrome and the entire comorbidity of ESRD. Main prevention measures are to preserve as longer as possible residual renal function in pre-dialysis patients and to identify, and if possible to treat properly, HMW accumulation and contributors such as carbonyl stress (Miyata et al., 2001), the presence of defects in the antioxidant status and $NO^{\bullet}$ metabolism (Asahi et al., 2000), recurrent infections and immune dysfunction, and above all improving dialysis quality (better material biocompatibility and use of ultrapure dialysate).

In the last years, innovative materials and improved dialysis techniques have been proposed with the end of developing strategies to prevent or dampen HD-related inflammation and oxidative stress. Promising results have been obtained using ''high performance dialysis techniques''. These include strategies based on mixed diffusive-convective methods, adsorption techniques (Cole et al., 2002; Mariano et al., 2005) and ultra-high flux protein-leaking dialysers (PLD) (Buoncristiani et al., 1999; Galli et al., 2001, 2003a, 2005a; Tessitore et al., 2004). Among high-flux synthetic membranes, PLD may provide a superior performance by direct removal and/or adsorption of HMWs. Polymethylmethacrylate (PMMA)-based membranes have been the most studied in this category. Importantly, only these and other electronegative membranes, such as polyacrylonitrile AN69 membranes, are commonly considered to have adsorption properties that increase the removal rate of some large solutes, cytokines, and middle molecules such as native and glycated β2-microglobulin (Randoux et al., 2001). Thus neutral membranes such as polysulfone (PS)-based dialysers, have been long considered to have negligible adsorption properties. Recent proteomic studies, however, suggested that several small and middle size solutes can be recovered after desorption also from PS dialysers (predominantly molecular masses lower than β2-microglobulin, i.e. 11,730 m/z units) (Ishikawa et al., 2006).

Extensive research by some of us showed that PMMA-based PLD significantly and steadily decrease pre-HD plasma levels of several protein damage markers included in the MW range in which these membranes exert maximal depurative efficacy. These markers evaluated over a 6-month follow-up study included total pentosidine (or AGE-pentosidine), AOPP, protein carbonyls, polyaminated proteins (Galli et al., 2001, 2005a), and protein-bound solutes such as Hcy (Galli et al., 2003a).

According with the evidence obtained by others (Tessitore et al., 2004), an intriguing aspect revealed by these studies is that the quota of each type of HMW removed and recovered in the dialysate (pentosidine and Hcy were evaluated in detail) was not sufficient to explain acute intra-HD decreases and the steady correction of pre-HD levels caused by PLD treatment. Neither can the quota of membrane adsorption (in the case of PMMA membranes) provide a sufficient contribution to explain this gap. This evidence suggested that a common mechanism is at the origin of the depurative effect that PLD exerts on a so heterogeneous group of large solutes. It was speculated that for some classes of large solutes, a steady correction of the pre-HD levels could be reached through the removal of limited, but biologically relevant, amounts of other and so far unidentified large solutes. These might work as triggers or early effectors of a common metabolic defect (possibly associated with oxidative and carbonyl stress, and SR recognition of modified proteins) that ultimately generates different classes of HMWs. Albumin with its relative abundance and multiple damage pathways is one of the best candidates for this role of early player. In fact, it is the most abundant proteinaceous solute removed by PLD (in the range of 1–4 g/dialysis session, an amount similar or even lower than the amount of proteins lost during peritoneal dialysis) (Galli et al., 2003a, 2005a), and it might contribute to produce a common mechanism of metabolic dysfunction through the generation of several modified forms and the stimulation of the SR response in the circulation and vessel components of tissues. In support of this hypothesis, during chronic inflammation, a defective albumin turnover may occur by impaired (IL-6-induced) de novo synthesis in liver cells and/or lower removal of modified forms by saturation of scavenging mechanisms (Fig. 1).

Accordingly, albumin is the main pentosidine-containing protein removed by PMMA-based PLD (Galli et al., 2005a), and immunoblotting experiments confirm that it predominates among the several ROS/RNS modified (3-nitro-tyrosine containing) plasma proteins recovered in dialysate specimens of patients treated with high flux dialysers (Galli et al. unpublished observation; see below). Other data based on the assay of protein carbonyls, further confirm the extensive damage of albumin besides other serum proteins of HD patients (Himmelfarb and McMonagle, 2001).

Therefore, the preliminary evidence on the depurative effects of PLD seem to be sufficient to stimulate further clinical research to verify the possibility that they could represent a tool useful in constraining inflammation of chronic HD patients. Other points in favour of the use of these dialysers are: 1) PLD are made of synthetic materials, and this is the highest standard of biocompatibility so far available; 2) these membranes can be used with standard dialysis techniques commonly adopted in the majority of HD centers without adverse effects associated with malnutrition and hypoalbuminemia or risk of backfiltration either in the short or long term (Galli et al., 2005a).

High-flux non-protein-leaking dialysers (NPLD), with nominal cut-off values usually below 30 kDa, are under evaluation for efficacy in removing circulating reactive carbonyls and middle molecules such as LMM-AGEs or β2-microglobulin in its native or glycated forms. This success may prevent protein damage and biological consequences of carbonyl stress (Randoux et al., 2001; Wanner et al., 2004; Bordoni et al., 2006). However, the fact that these membranes do not remove efficiently larger molecules could represent a major limit of performance. To address this limitation, it has been proposed to adopt mixed diffusive-convective methods also implemented by the use of specific materials such as sorbent resins, which are under preliminary evaluation to set innovative hemoperfusion techniques (Winchester et al., 2002).

Pharmacological approaches to break the inflammatory loop of HD patients can be based on AGE inhibitors or disruptors such as aminoguanidine (Thornalley, 2003). This drug and others, such as ALT-711, inhibit AGE-induced heart hypertrophy or stiffness of the large arteries (Simm et al., 2004). At the same time, aminoguanidine and metformin prevent impairment of HDL-mediated cell cholesterol removal in peritoneal macrophages and in SR-BI transfected cells induced by AGE-albumin and cell glycoxidation. Such a biological event could facilitate the development of premature atherosclerosis in diabetes mellitus and other disease states associated with carbonyl and oxidative stress (Machado et al., 2006). However, these drugs are still far from being approved for clinical use. At present, safety concerns and apparent lack of efficacy have stopped the use of aminoguanidine in clinical trials on diabetic nephropathy (Thornalley, 2003).

Proteomics of HMWs

The scarce knowledge on the nature and biological significance of proteinaceous solutes contained in uremic blood limits the possibility to interpret their role as uremic toxins and the efficacy of dialysis techniques to correct accumulation. In this context, proteomic analyses of serum and spent dialysis fluid proteins can provide a key tool for systematic molecular characterization of uremic HMWs.

Recently a first attempt to identify ultrafiltrate proteins was performed (Molina et al., 2005). Dialysis fluid enriched with proteins showing a MW between 10 and 50 kDa was obtained from a patient with acute renal failure treated with a polyacrylonitrile-based AN69 membrane (nominal cut-off < 50 kDa, i.e. a NPLD). The choice to study ultrafiltrate proteins obtained by NPLD, instead of whole serum proteins, was adopted as an elective criterion to deplete high MW proteins (mostly albumin) that can interfere with protein and peptide analysis with relative concentrations set in the lowest range. The electrophoretic separation of ultrafiltrate proteins followed by in-gel digestion and LC-MS/MS analysis allowed identification of 292 proteins, 205 of which had never been reported (identified) in human serum or plasma. Additional biochemical and immunoenzymatic analysis revealed that many of these had never been identified previously because of their low concentrations. Several proteins showed post-translational modifications useful to map mature forms of N-termini. In vivo proteolytic fragmentation was tentatively identified in some cases. New proteins, including cytokines, have been identified as predicted transcripts on data bases.

In another proteomic study by the group of Vanholder (Weissinger et al., 2004), the analysis of peptide maps of ultrafiltrates generated with low-flux (LF) and high-flux (HF) polysulfone-based dialysers was performed after albumin removal and capillary electrophoresis coupled with MS spectrometry, which allowed to investigate solutes with a MW up to 10 kDa. The analysis of ulrafiltrates from uremic plasma obtained from HF and LF showed 1394 and 1046 polypeptides, respectively. These numbers were reduced by more than a half when ultrafiltrates prepared with healthy plasma samples were assessed. Only few proteins were tentatively identified by MALDI-TOF-TOF analysis in this study, which in turn demonstrated the superiority of HF as compared with LF membranes in removing middle and large polypeptides.

The MALDI-TOF-TOF study carried out by Lefler et al. (2004) on ultrafiltrate proteins from an acute renal failure patient, identified 10 different proteins including albumin, apolipoprotein A-IV, beta-2-microglobulin, lithostathine, mannose-binding lectin associated serine protease 2 associated protein, plasma retinol-binding protein, transferrin, transthyretin, vitamin D-binding protein and Zn alpha-2 glycoprotein.

Recently protein removal and adsorption have been evaluated by SELDI-TOF-MS and ProteinChip array in patients treated with PS and PMMA moderate flux membranes (both are included in the category of NPLD) (Ishikawa et al., 2006). Mass-to-charge ratios (m/z) between 2000 and 120,000 were analyzed. β2-microglobulin (identified as m/z 11,730) was predominantly adsorbed onto, and only a small amount was filtered throughout, PMMA membranes, which in total were found to adsorb 149 polypeptides. Sixty-eight peptides were adsorbed more by PS than PMMA membrane and the majority of them showed m/z values less than 11,730. Dominant peaks adsorbed onto PS showed m/z of 6629 and 6431 and were identified as apolipoprotein CI and truncated apolipoprotein CI, respectively. Thirty-seven proteins with MW > m/z 11,730 showed greater filtration through PMMA than PS.

These early approaches clearly shows that proteomic studies can provide crucial information on the characteristics of proteinaceous solutes present in uremic blood and removed by dialysis procedures (applied either to acute or chronic treatments). This new and particularly appealing field of investigation remains yet largely unexplored.

Our group has performed a series of comparative analyses of plasma and ultrafiltrate samples obtained from patients treated with PMMA-based PLD (BK-F type, nominal cut-off 70 kDa, Toray, Japan) and standard polysulfone-based high-flux NPLD (Piroddi et al., unpublished observation). In the latter case, two types of membranes were used: FX-80 dialysers (Fresenius, Germany) identified as NPLD1 and Toraysulfone dialysers (Toray, Japan) identified as NPLD2. Consistent with previous experiments (Galli et al., 2005a), it has been established that these two membranes produce a net protein leakage per dialysis session of approximately 3 and <0.3 g, respectively. Using one-dimensional gel electrophoresis analysis (1D-PAGE) on the same amount of total proteins (20 μg/gel line) as a first qualitative and semi-quantitative analysis approach to compare individual ultrafiltrate proteins, major differences were observed between the two classes of dialysers (Fig. 3). Qualitative differences confirmed with the Quantity-One software (Bio-Rad) (not shown) allowed to demonstrate that, in accordance with the nominal cut-off declared by the manufacturer, protein leakage of PLD was represented mainly by proteins with MW ≥ 60 kDa, albumin representing the predominating band in PAGE analyses. In the case of NPLD, it was difficult to confirm the cut-off declared by the manufacturers, since proteins recovered in the dialysate showed a broad MW range although with MW predominantly

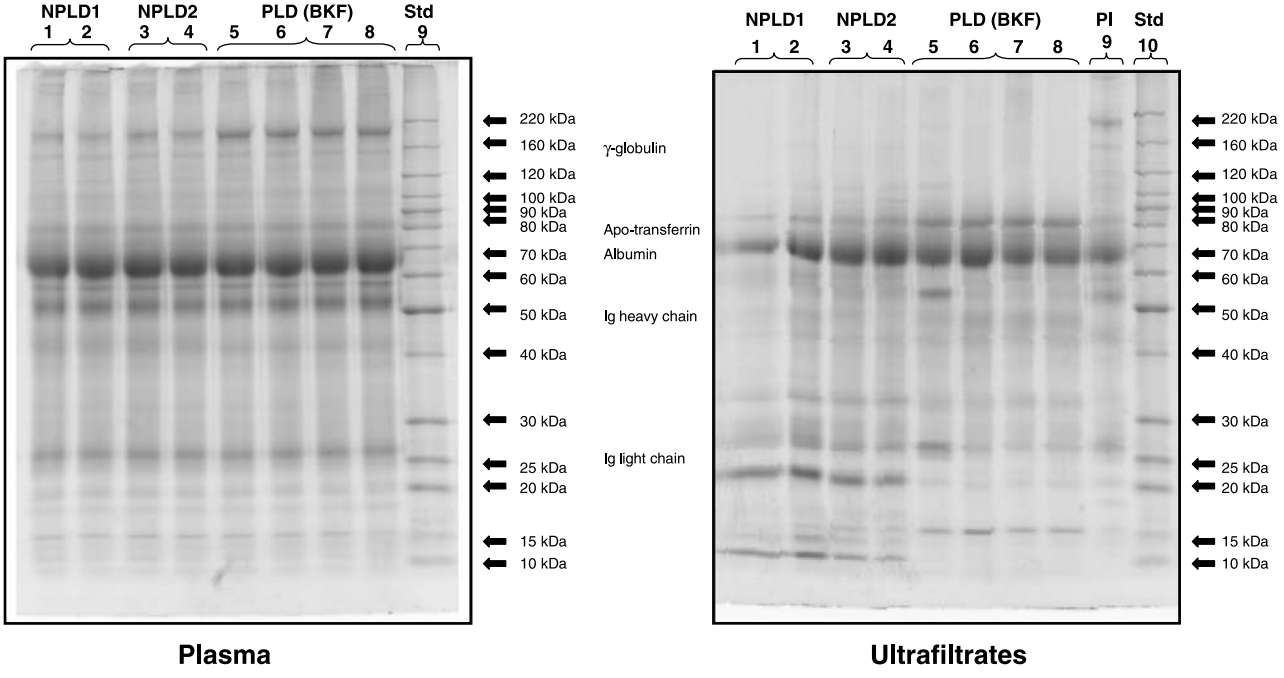

Fig. 3. Monodimensional SDS-PAGE gradient analysis (%T range = 9–16) of the protein pattern in plasma (left panel) and ultrafiltrate (right panel) samples from subjects treated with two types of polysulfone-based non protein-leaking dialysers (NPLD: *1–2* and *3–4*) and polymethylmethacrylate-based protein-leaking dialysers (PLD, BKF series: *5–8*). For comparison with ultrafiltrate protein patterns, a sample of plasma is shown also in the right panel (*9*). Samples loaded in all the lanes corresponded to 20 μg of total proteins. Qualitative and semi-quantitative image analysis of 1D patterns was performed with Quantity-one 1-D analysis software (Bio-Rad)

lower than that of albumin. These characteristics in protein patterns of NPLD ultrafiltrates clearly demonstrate that, in contrast to PLD, protein leakage is non-specific, although limited as an entity.

To investigate the presence of damaged proteins in uremic ultrafiltrates obtained with PLD and NPLD, proteins separated by 1D-PAGE were electroblotted and immunostained with antibodies against the hallmark of RNS-dependent and inflammation-related protein damage, 3-nitrotyrosine (Fig. 4). The results suggested the presence of multiple modifications in a large number of proteins, thus confirming the extensive damaging effects of the uremic and oxidative environment on plasma proteins, and previously proposed thanks to the analysis of protein carbonyls in uremic serum (Himmelfarb and McMonagle, 2001). In comparison to uremic serum proteins, our preliminary data confirmed that albumin is a preferential target of nitration together with transferrin. Other bands in the MW region 10–30 kDa were stained and together with unidentified proteins, these seem to include the IgG light chain as one of the most abundant immunoreactive

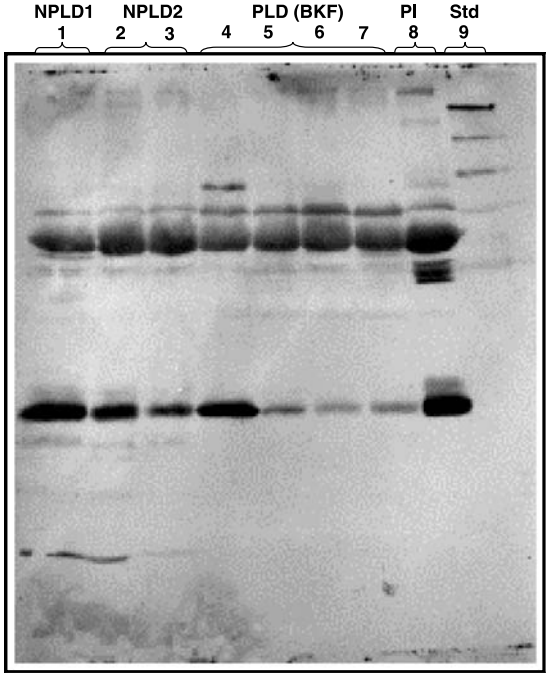

Fig. 4. Western blotting analysis of anti-3-nitrotyrosine immunoreactive proteins in ultrafiltrate samples from subjects treated with two types of polysulfone-based non-protein-leaking dialysers (NPLD: *1* and *2–3*) and polymethylmethacrylate-based protein-leaking dialysers (PLD, BKF series: *4–7*). For comparison with ultrafiltrate proteins, a sample of uremic plasma was also assessed (*8*). Proteins (100 μg) were separated after 1D-PAGE and after electroblotting on a nitrocellulose sheet were immunostained with a mouse monoclonal anti-3-nitrotyrosine antibody (Upstate, Lake Placid, NY, USA) and subsequently with goat anti-mouse antibody – HRP conjugate (Upstate, Lake Placid, NY, USA)

elements. Faint signals were observed for proteins with higher MW such as fibrinogen (see the line at the origin; MW = 340 kDa), which was reported to be extensively carbonylated in uremic serum (Himmelfarb and McMonagle, 2001). Again main differences were observed in the profile of 3-nitro-tyrosine containing proteins of NPLD and PLD ultrafiltrates. Interestingly, the two types of PS-based NPLD showed different bands in the low MW area, and PLD were effective in removing a large amount of modified proteins. All bands immunostained in the different MW regions of this WB analysis are under evaluation for 2D analysis and specific identification by LC-MS/MS. Although these are only preliminary results, they clearly show how proteomic analysis performed with immunodetection of modified proteins [the study of 3-nitro-Tyr containing proteins is an example of applied ''redoxomics'' (Dalle-Donne et al., 2006)] could be used to characterize the presence of HMWs and to assess dialysis membrane performance.

Two-dimensional electrophoresis experiments were preliminarily carried out on ultrafiltrate proteins (Fig. 5), without removing albumin, and protein pattern analysis was performed using PD-Quest 2D gel analysis software (Bio-Rad). Tentative identification of individual proteins was performed by matching 2D maps of NPLD and PLD ultrafiltrates (Fig. 5A and B, respectively) with plasma protein maps available in the SWISS-PROT database (Fig. 5C) of the EXPASY web site (http://www.expasy.org/cgi-bin/map2/def?PLASMA_HUMAN). A maximum of 314 and 230 spots were detected in NPLD and PLD ultrafiltrates, respectively. The difference between the two types of membranes can be defined as a size-exclusion effect, which in the case of NPLD allows to achieve a higher relative abundance of proteins by albumin exclusion (this may thus represent a strategy to obtain albumin depletion). On the contrary, in the case of PLD, albumin is massively removed and we suggest that it exerts a sort of mass action on membrane pores, which ultimately may limit the passage of other solutes. Therefore, for several of these solutes, the relative concentration decreased below the detection limit imposed by the staining procedures used to visualize protein spots onto 2-D maps. In this case, selective albumin removal by immunoaffinity or chromatographic methods may lead to increase the number of detectable spot and thus to tentatively identify other uremic proteins either in the low or high MW range. At the same time, however, albumin removal can introduce a methodological bias since it produces a heavy loss of several proteins. This can lead to change relative concentrations and drastically compromises semi-quantitative analyses.

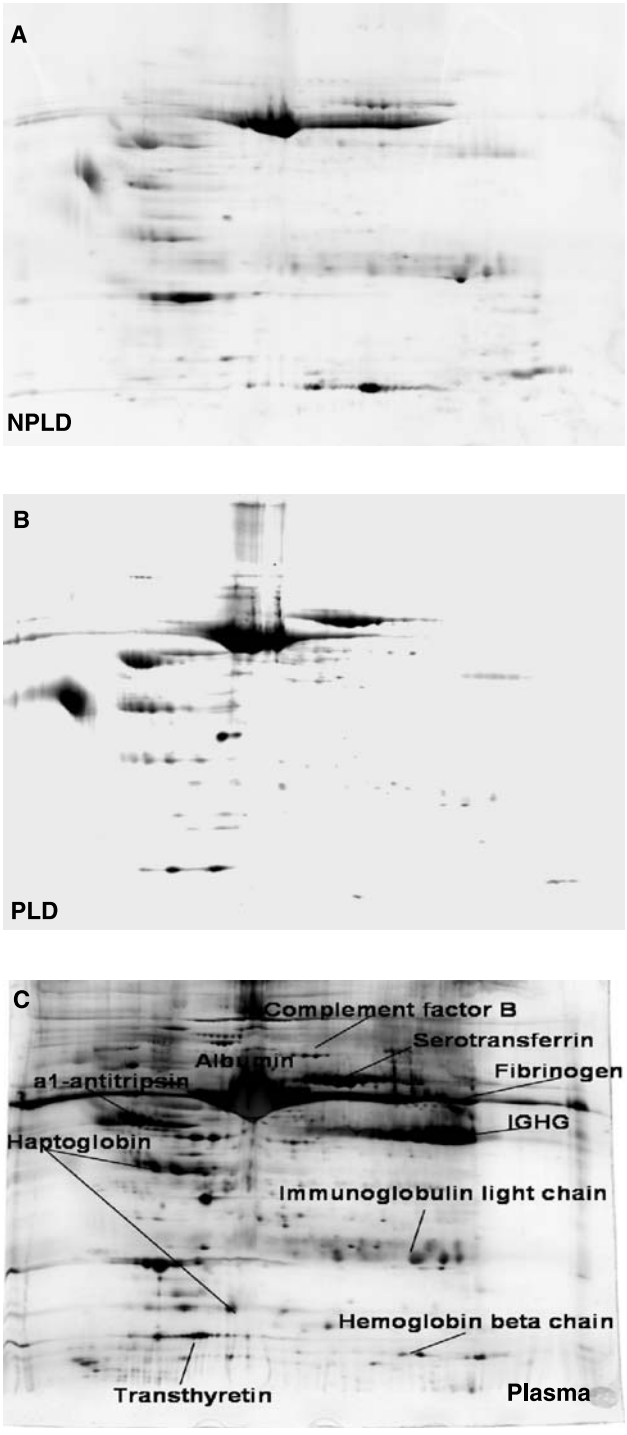

Fig. 5. Bidimensional SDS-PAGE gradient electrophoresis (%T range = 9–16) of ultrafiltrate proteins from one patient treated with NPLD2 (**A**; corresponding to the sample loaded in lane 4 of Fig. 3, right panel) and one patient treated with PLD BKF series (**B**; corresponding to the sample loaded in lane 7 of Fig. 3, right panel). **C** shows a corresponding 2-D SDS-PAGE gradient electrophoresis (%T = 9–16) of plasma proteins in a healthy subject reported in the SWISS-PROT database at the EXPASY web site (http://www.expasy.org/cgi-bin/map2/def?PLASMA_HUMAN). In the analyses of ultrafiltrate and plasma proteins 200 and 700 μg of total proteins were loaded, respectively

Preliminary semiquantitative analysis (by gel matching) revealed that from protein spots detected in PLD ultrafiltrate samples, 88 were not present in healthy control plasma, thus representing proteinaceous solutes possibly related to uremia and dialysis. Thirty-six proteins identified in ultrafiltrate samples were not reported (or were not identified) in the SWISS-PROT database. These may represent newly identified plasma proteins, which according to the observation of Molina et al. (2005) become evident on 2-D PAGE analysis due to the concentration (size-exclusion) effect during blood ultrafiltration.

In the comparison of whole ultrafiltrate 2-D patterns of PLD and NPLD there were differences in either spot presence/absence or signal intensity (as 2-fold difference) in 143 proteins.

Ongoing research will provide further details on protein identification by 2-D map matching or LC-MS/MS and sequencing analyses.

Conclusion

Pro-inflammatory solutes accumulate in uremic blood and may contribute to the onset and progression of ESRD inflammatory syndrome. Carbonyl and oxidative stress, together with overall metabolic alterations induced by the absence of kidney function, may contribute to uremic intoxication through damage of plasma and tissue proteins. As a consequence, the uremic condition is associated with the formation and accumulation in the circulation of a heterogeneous class of proteinaceous solutes (or HMWs). Molecular and biochemical data are consistent with the evidence that these solutes mainly originate from the oxidation and/or glycation of specific residues of serum and tissues proteins. The binding of small molecules deriving from carbonyl and oxidative stress can provide also a substantial contribution to protein modification.

In vitro data and early clinical evidence suggest that HMWs reach levels in the circulation and exert biological functions that could be relevant to sustain several aspects of ESRD comorbidity. The mechanism proposed for the pathogenic role of these aberrant solutes is related to SR-dependent recognition and activation of inflammatory and vascular cells which in turn sustain inflammation, vascular dysfunction and chronic-degenerative events in different organs and tissues. This SR-dependent mechanism can contribute to generate a self-feeding inflammatory loop in which HMW generation/accumulation play a central role. For this reason, HMWs can be defined as true uremic toxins.

In the circulation oxidatively modified and glycated forms of albumin quantitatively predominates among other damage protein products so far identified in this heterogeneous class of proteinaceous solutes. These forms of albumin are among the most relevant SR ligands and pro-inflammatory triggers, and therefore they could play a major role in the onset and feeding of the inflammatory loop of ESRD.

Transplantation seems to almost completely solve the problem of HMW accumulation and toxicity. Substitutive techniques based on filtration and/or adsorption methods are under evaluation to develop strategies to reduce the pro-inflammatory burden of uremic blood. Pharmacological approaches are based on drugs that may decrease reducing sugar and free carbonyl reactivity ultimately preventing the formation of proteins adducts. However, in spite of encouraging in vitro results, clinical application of the most promising drugs seems to be far from becoming a reality due to issues regarding toxicity and efficacy. Antioxidant therapy might help to prevent oxidative processes that act as underlying events in protein damage. However, there is only limited evidence to support this and more investigation is warranted.

Further studies are needed to understand the biological and clinical meaning of HMW accumulation in ESRD. Advanced biochemical and molecular techniques (such as proteomis of serum and ultrafiltrate solutes) now offer a precious tool to better characterize these proteinaceous solutes and their impact on the homeostasis of inflammatory pathways and vascular function, as well as on other relevant aspects of general comorbidity of ESRD.

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