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Uremic toxins modulate the spontaneous apoptotic cell death and essential functions of neutrophils

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Uremic toxins modulate the spontaneous apoptotic cell death and essential functions of neutrophils. Clearance of neutrophils via apoptosis from the site of infection is crucial for the coordinated resolution of inflammation. The balance between stimulating and attenuating as well as between pro- and anti-apoptotic factors is necessary for maintenance of an effective immune response without the harmful side effects of neutrophil action. This article describes the effect of glucose-modified serum proteins and of free immunoglobulin light chains (IgLCs) on neutrophil functions and apoptosis. Both groups of proteins are found at elevated levels in sera of uremic patients. Glucose-modified proteins increase both the chemotactic movement of neutrophils and the activation of glucose uptake. Spontaneous neutrophil apoptosis is increased in the presence of these modified serum proteins. On the other hand, the presence of free IgLCs, previously shown to diminish neutrophil chemotaxis and the activation of glucose uptake, increase the percentage of viable neutrophils by inhibiting spontaneous apoptotic cell death. We conclude that both glucose-modified proteins and free IgLCs can be considered to be uremic toxins and both contribute to the disturbed immune function in uremic patients. Their concentrations as well as the microenvironment in which they are acting seem to be important for their actual effects.

Bacterial infections still represent a main cause for the increased morbidity and mortality among uremic patients [1, 2], mainly as a result of the altered functions of neutrophils [3]. Neutrophils are cells of the first-line unspecific immune defense. After the chemotactic movement to the site of infection, their main job is to destroy invading microorganisms after phagocytosis by secretion of proteolytic enzymes and the generation of reactive oxygen metabolites during the oxidative burst. Any modulation of one of these essential functions will lead to an increased risk for bacterial infections. Neutrophils isolated from uremic patients show functional changes such as a reduced chemotactic activity [4], a lower cellular response to phagocytic stimuli [5] and a diminished oxidative metabolism leading to disturbed intracellular killing [6]. This contributes to the higher risk of bacterial infections. Factors responsible for the altered neutrophil functions have been described in the literature: anemia, malnutrition, iron overload, zinc deficiency, increased levels of intracellular calcium, and hemodialysis treatment per se [7]. Within the last few years the important role of uremic toxins in contributing to the diminished immune function in uremia has been recognized. A number of factors from hemodialysis ultrafiltrate and continuous ambulatory peritoneal dialysis (CAPD) effluents has already been isolated and characterized [8–14].

Killing of invading microbes is a precondition for a successful immune response. However, the discharge of neutrophil toxic products into the extracellular space leads to a prolonged inflammation and as a consequence to the destruction of the surrounding tissue [15]. Therefore, for a normal resolution of inflammation the clearance of neutrophils by macrophages from the site of infection is highly important when their plasma membrane is still intact. Recently, the importance of apoptosis, that is, programmed cell death, of neutrophils has been recognized. Human neutrophils have a short half-life and are already programmed to die via apoptosis when they enter circulation [16]. Apoptotic neutrophils that still have an undamaged cell membrane are recognized and taken up by macrophages and by semiprofessional phagocytes such as glomerular mesangial cells without the release of pro-inflammatory cytokines [17–19]. Apoptosis is an active process characterized by chromatin condensation, DNA cleavage at internucleosomal sites and loss of membrane asymmetry but not integrity. On the other hand, necrosis, the other main form of cell death, is a passive degenerative phenomenon characterized by the loss of the integrity of the cell membrane without changes in the morphology of the nuclei. The balance between neutrophil apoptosis and necrosis plays a role in the control of inflammation, and is important to avoid the development of inflammatory diseases [20].

The rate of neutrophil apoptosis seems to be regulated by death factors as well as by survival factors. Despite the importance of functioning neutrophil apoptosis at the site of inflammation, a general increase in neutrophil

Key words: immunoglobulin light chains, glucose uptake, uremia, bacterial infection, immune defence, chemotactic activity.

Table 1. Effect of glucose-modified proteins on neutrophil chemotaxis (relative distance migrated) and relative activation of glucose uptake in the presence of buffer (Co), unmodified serum proteins (S−), serum proteins modified with glucose alone (S+) or with glucose in the presence of aminoguanidine (S+ AG), and in the presence of glycated proteins isolated from CAPD effluents using boronate affinity chromatography (boronate-binding proteins, BBP)

	Co	S−	S+	S+ AG	BBP
Chemotaxis	100 ± 0	107 ± 6	130 ± 11	101 ± 9	ND
P			= 0.01 vs. S−	= 0.01 vs. S+	
Glucose uptake	100 ± 0	99 ± 13	135 ± 13	144 ± 23	164 ± 7
P			<0.005 vs. S−	<0.01 vs. S−	<0.005 vs. S−

apoptosis will lead to a disturbed immune response. Recently, it could be demonstrated that neutrophils obtained from uremic patients undergo accelerated apoptosis under in vitro conditions [21] and that constitutive cellular factors contribute to this effect. On the other hand, uremic plasma has also been shown to accelerate neutrophil apoptosis [21].

Unlike for essential neutrophil functions, no uremic toxin with the potential to modulate neutrophil apoptosis has been described so far. Recently, we identified factors that accumulate in uremic sera, modulate essential neutrophil functions and have a significant influence on the spontaneous apoptotic cell death of neutrophils (G. Cohen et al, manuscript submitted for publication), namely glucose-modified proteins and free immunoglobulin light chains (IgLCs).

EFFECTS OF GLYCATED SERUM PROTEINS ON NEUTROPHIL FUNCTIONS

Serum proteins obtained from healthy donors were modified with glucose in vitro. Furthermore, we wanted to investigate whether early or late glycation products are responsible for the effects of the modified proteins. As the compound aminoguanidine inhibits the advanced glycation end product (AGE) formation [22], but does not prevent the formation the early glycation products, we incubated serum proteins in the presence of glucose with and without the addition of aminoguanidine to obtain early and a mixture of early and late glycation products, respectively.

The effect of the modified serum proteins on the chemotactic movement of neutrophils was tested using the under-agarose method. Neutrophil chemotaxis was higher in the presence of modified serum proteins (S+) as compared to the unmodified control samples (S−; Table 1). This effect was not observed when serum proteins modified with glucose were added in the presence of aminoguanidine (S+ AG; Table 1). Therefore, late glycation products are responsible for the stimulatory effect on neutrophil chemotaxis.

The uptake and accumulation of 2-deoxy-D-[1−^{3}H]-glucose serves as a quantitative measurement of the state of activation of phagocytic cells [23]. The relative activation of the glucose uptake was significantly higher in the presence of the glucose-modified serum proteins (S+) than in the presence of unmodified proteins (S−; Table 1). Early and not late glycation products are responsible for this effect, because serum proteins incubated in the presence of glucose and aminoguanidine (S+ AG) showed the same effect on the activation of glucose uptake as proteins modified with glucose alone (Table 1).

EFFECTS OF GLYCATED SERUM PROTEINS ON SPONTANEOUS APOPTOTIC CELL DEATH OF NEUTROPHILS

We investigated the effect of the glucose-modified proteins on spontaneous neutrophil apoptosis by detecting the characteristic morphological changes and characteristic DNA-strand breaks. Apoptotic neutrophils are removed by phagocytes under in vivo conditions. Therefore, we did not distinguish between apoptotic and secondary necrotic cells, and show the percentage of viable cells in the results of the apoptosis assays. As shown in Table 2, neutrophils die faster in the presence of glucose-modified proteins as compared to the controls. We also used individual modified proteins, such as human serum albumin, in our apoptosis assays and could confirm the results obtained with whole serum proteins (Table 2). As observed for the effect on the uptake of glucose, early and not late glycation products are responsible for the effects observed, because proteins incubated in the presence of aminoguanidine show the same effect on spontaneous neutrophil apoptosis as proteins incubated in the presence of glucose alone (Table 2). There are high expectations towards aminoguanidine in terms of ameliorating AGE-related disturbances such as diabetes associated vascular hypertrophy [24]. However, the results from our in vitro experiments suggest that aminoguanidine is not able to prevent the increased neutrophil apoptosis caused by glycated proteins.

Our findings are also relevant for the status of the immune system in diabetic patients with increased plasma AGE concentrations. Interestingly, it has been suggested that AGEs play an important role in the development of microvascular complications in diabetes mellitus by inducing apoptosis and increasing pro-coagulant activity

Table 2. Effect of glucose modified proteins on neutrophil apoptosis

	Co	S−	S+	S+ AG	A−	A+	A+ AG	BBPs	S
% Viable neutrophils	32 ± 3	51 ± 4	44 ± 4	45 ± 4	42 ± 7	34 ± 5	32 ± 3	43 ± 3	65 ± 3
P			<0.005 vs. S−	<0.05 vs. S−		<0.05 vs. A−	<0.05 vs. A−	<0.0005 vs. S	

Percentage of viable cells after a 20-hour incubation in the presence of: buffer (Co), serum proteins (S−) or human serum albumin (A−) incubated without glucose; serum proteins (S+) or albumin (A+) modified with glucose alone or with glucose in the presence of aminoguanidine (S+ AG, A+ AG); glycated proteins isolated from CAPD effluents (boronate binding proteins, BBPs) and unmodified serum protein(s).

in umbilical vein endothelial cells [25]. Furthermore, it has been demonstrated that in diabetic patients there is a neutrophil activation and that this activation is correlated to glycated hemoglobin level [26].

As no AGE-binding proteins have been described for neutrophils to date, the mechanism of the effect of glycated proteins still remains to be elucidated. However, receptors for AGEs have been detected on monocytes [27] and endothelial cells [28] where they play a role in the removal of glycated proteins and in changes of gene expression [29]. For example, fluorescent AGE-β2-microglobulin but not unmodified β2-microglobulin exhibits biological activity with monocytes/macrophages [30].

Our results are consistent with the finding of Cendoroglo et al that neutrophil apoptosis is increased in uremia [21]. The same authors found diminished functions of apoptotic neutrophils. However, we investigated the immediate effect of protein samples on normal neutrophils, whereas Cendoroglo et al determined the functions of neutrophils that are already undergoing apoptotic cell death. They concluded that uremia in neutrophils enhances some functional activities such as oxidative metabolism at an early phase leading to functional impairment of neutrophils in a later phase [21]. This is also consistent with the observation that the dysregulation of the immune system in uremia is characterized by immune deficiency, even though the immune competent cells exist in a state of activation [31]. Another group of modified proteins, advanced oxidation protein products (AOPP), has a close correlation to AGEs [32]. Both AOPP and AGEs have significantly elevated plasma levels in uremic patients and both protein modifications confer features on proteins that lead to the stimulation of the oxidative burst of human monocytes in vitro [32]. It has also been shown that neutrophils from uremic patients are primed for an enhanced oxidative metabolism [33], and that this primed state could not be changed by hemodialysis or continuous ambulatory peritoneal dialysis (CAPD) treatment [34].

EFFECTS OF GLYCATED PROTEINS ISOLATED FROM UREMIC PATIENTS ON NEUTROPHIL FUNCTION AND SURVIVAL

We showed that the observed effect of glycated proteins is not restricted to in vitro modified samples, as glucose-modified proteins isolated from the effluent of CAPD patients increase the relative activation of glucose uptake significantly (Table 1). Furthermore, we demonstrated that glucose-modified proteins that were isolated from CAPD effluents significantly reduce the number of surviving polymorphonuclear leukocytes (PMNL) as compared to the controls (Table 2). As it has been shown that the presence of proteins per se is able to modulate PMNL apoptosis, serum proteins of healthy donors at the same final concentration have been used as controls in this set of experiments.

INFLUENCE OF FREE IGLCs ON NEUTROPHIL FUNCTIONS

Plasma levels of free Ig light chains are elevated in patients with impaired kidney function. A significant increase in the levels of both κ and λ chains in anephric patients has been described by Solling [35]. Wakasugi et al reported on the increase in the concentration of free Ig light chains in sera after the start of hemodialysis therapy [36]. Using a newly developed assay based on SDS-PAGE, electrotransfer onto nitrocellulose membranes, and chemiluminescence detection, our group found increased levels of Ig light chains in uremic predialysis patients as well as in chronic renal failure patients undergoing hemodialysis treatment (Cohen et al, manuscript submitted). Furthermore, we showed that the light chain levels could not be significantly decreased by either low-flux or high-flux dialyzer membranes. We previously showed that free IgLCs isolated from hemodialysis and CAPD patients as well as commercially available Bence Jones proteins significantly inhibit chemotaxis and glucose uptake of neutrophils [10]. We conclude that free IgLCs are at least partly responsible for the diminished unspecific immune defense and consequently for a higher risk of infection in uremia.

INFLUENCE OF FREE IGLCs ON NEUTROPHIL APOPTOSIS

Using the same assays as described above for glucose-modified proteins, we could show that Ig light chains of both κ-and λ-type increase the percentage of viable

Table 3. Effect of free immunoglobulin light chains (IgLCs) on neutrophil apoptosis

Specific antibody	Co	κ	λ	A
Absent	12 ± 1	38 ± 8*	36 ± 7*	21 ± 3
Present	ND	12 ± 4	17 ± 3	17 ± 6

Percentage of viable cells after a 20-hour incubation in the presence of buffer (Co), IgLCs of κ- or of λ-type and of human serum albumin (A) in the absence or presence of the respective specific antibody specific antibody. *$P < 0.05$ versus Co.

neutrophils by diminishing apoptotic cell death (Table 3) in a concentration dependent manner (G. Cohen et al, manuscript submitted for publication). The effect of the IgLCs was specific as demonstrated by the fact that this effect can be abolished by specific antibodies against κ and λ light chains, respectively (Table 3). The presence of human serum per se is able to decrease neutrophil apoptosis [5]. However, there are three lines of evidence showing that our results are not solely based on a protein effect, but are specific for Ig light chains: (*1*) the addition of antibodies should increase and not abolish a protein effect; (*2*) the Ig light chain concentration used for the apoptosis experiments is much lower than the concentration of serum proteins leading to the same percentage of surviving neutrophils; and (*3*) in the presence of excess serum proteins κ and λ light chains inhibit neutrophil apoptosis as well, whereas albumin exerts no significant effect under the same experimental setup (G. Cohen et al, submitted for publication).

As discussed above, free IgLCs diminish essential neutrophil functions. This effect is at least partly caused by a prestimulation of neutrophils, for example an increased basal level of glucose uptake [10]. It is likely that IgLCs are able to contribute to the chronic state of inflammation found in end-stage renal disease patients [31] if they are also able to prolong the life of these pre-activated neutrophils that have shown to play an important role in tissue injury and the development of renal failure [15].

It is important to keep in mind that there seems to be a physiological balance between anti-apoptotic and counteracting pro-apoptotic factors, and that the microenvironment in which they are acting has to be considered. Freely circulating IgLCs, for example, will contribute to the fate of neutrophils in a different way than accumulated light chains as observed in the light chain deposition disease. Furthermore, it has been shown that neutrophils that transmigrated through endothelial monolayers have altered functional properties and have a different tendency to undergo apoptosis [37]. Reduced [38, 39] as well as increased neutrophil apoptosis has been described for PMNL after the migration across an endothelial monolayer. It also has been shown that extracellular matrix proteins regulate local inflammations by influencing apoptotic cell death in tumor necrosis factor-α (TNFα)-activated neutrophils [40]. On the other hand it has been described that neutrophils are protected against apoptosis in circulation by red blood cells acting as scavengers of apoptosis promoting H_2O_2 [41]. In vitro experiments demonstrated that the extracellular pH modulates the rate of neutrophil apoptosis, alkaline conditions accelerating [42] and extracellular acidosis depressing [43] apoptosis.

In conclusion, uremic toxins accumulating in the plasma of patients with renal failure not only affect essential neutrophil functions and thereby the unspecific immune response, but also influence neutrophil survival by modulating the rate of apoptotic cell death. Identifying factors influencing both function and apoptosis of neutrophils is a first step in understanding the causes for the deranged immune system in uremia.

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