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In vitro evidence for immune activating effect of specific AGE structures retained in uremia

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In vitro evidence for immune activating effect of specific AGE structures retained in uremia.

Background. Advanced glycation end-products (AGEs) have been identified to be accumulated in blood and tissues of patients with end-stage renal disease (ESRD). AGEs have been shown to modulate immune competent cell activities and in this way they may contribute to the progression of atherosclerosis. All studies in this context have been performed, however, with generated mix of glycation compounds, and not with structures similar to those encountered in uremia. In the present study, the immunologic effect of specific AGE compounds, known to be retained in uremia, has been evaluated.

Methods. Four albumin preparations, modified chemically at lysine or arginine residues, respectively, to contain N-ε-carboxymethyllysine (CML albumin), N-ε-carboxyethyllysine (CEL albumin), glyoxal-induced imidazolinones (Arg I albumin) or methylglyoxal-induced imidazolinones (Arg II albumin) were applied. Their effect on chemiluminescence production, CD14 expression, and the DNA synthesis of calcitriol-differentiated HL-60 (monocyte/macrophage phenotype) was studied.

Results. The phorbol 12-myristate 13-acetate (PMA)-stimulated chemiluminescence production of the calcitriol differentiated HL-60 cells was enhanced in the presence of CEL albumin (44.1 ± 18.5 vs. 64.7 ± 28.1 counts 10^3/30 min) ($P < 0.05$), Arg I albumin (46.4 ± 18.8 vs. 66.1 ± 32.6 counts 10^3/30 min) ($P < 0.05$) and CML albumin (41.9 ± 25.5 vs. 60.9 ± 5.5 counts 10^3/30 min) ($P = 0.0625$) pointing to an increase in free radical production. The latter AGE compounds also significantly increased the calcitriol-induced CD14 expression on HL-60 cells (1675 ± 796 vs. 2075 ± 1044; 768 ± 143 vs. 890 ± 150; 647 ± 63 vs. 716 ± 69 mean fluorescence intensity) ($P < 0.05$, respectively) pointing to an increase in expression of the lipopolysaccharide (LPS) receptor. Finally, the DNA synthesis of the calcitriol-differentiated HL-60 cells was enhanced in the presence of Arg I albumin [34.5 ± 4.6 vs. 27.7 ± 9.7% 5-bromo-2′-deoxyuridine (BrdU)-positive cells] ($P < 0.05$) resulting in an increased cell proliferation.

Conclusion. Genuine AGE compounds, as they are encountered in the uremic condition, activate leukocyte response, and hence could play a role in uremia related atherogenesis.

Key words: Advanced glycation end products, uremia, immune activation.

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Until now, the research of the biologic effects of advanced glycation end-products (AGEs) has only been performed with mixtures of AGEs, obtained after a long lasting in vitro preparation process, whereby glucose is brought into contact with albumin at a temperature of 37°C in an aspecific way, resulting in "AGE-modified" proteins containing several peptide-bound amino acid derivatives of unknown concentration [1–3]. To the best of our knowledge, no such studies have been undertaken until now with proteins containing genuine AGE compounds, as they are encountered in the serum of uremic patients. Hence it remains unclear which of the known retained AGE products, and to what extent, exert biologic effects.

AGE compounds are generated through chemical rearrangements and degradation reactions from stable Amadori products and Schiff-base adducts which are the result of a nonenzymatic reaction of glucose or reducing sugars with free amino groups [4]. Next to the generation of AGE compounds in the process of aging [5] and during the progression of diabetes mellitus [5, 6], AGEs were also identified in the blood and tissues of patients with end-stage renal disease (ESRD). Oxidative and carbonyl stress were pointed out as responsible actors in uremia, rather than reactions with glucose [7]. It was recently demonstrated that AGEs accumulate in atheromatous plaques of the aortic wall of subjects with ESRD, where they may further contribute to a more rapid progression of atherosclerosis [8]. AGEs can also neutralize the process that protects the vascular bed against aggregation and proliferation of cells, two key elements in atherogenesis. Most in vitro work has shown AGEs to be part of complex interactions within the context of oxidative stress and vascular damage, particulary in atherosclerosis [9] and in the accelerated vascular damage that occurs

in diabetes, as well as in other conditions such as ESRD [10, 11].

The purpose of our study, therefore, was to evaluate changes due to the presence of individual, chemically characterized AGE compounds, which are known to be retained in renal failure, and play a possible role in immune dysfunction and atherogenesis. For that purpose, the albumin-modified AGE compounds N-ε-carboxymethyllysine (CML albumin), N-ε-carboxyethyllysine (CEL albumin), arginine modified with glyoxal (Arg I albumin) and arginine modified with methylglyoxal (Arg II albumin) were chosen.

CML may result from the reaction of lysine side chains with glyoxal [12] and the oxidative cleavage of fructosamines [13]. Plasma levels of CML in hemodialysis patients by far exceed those in normal or diabetic subjects, possibly due to increased carbonyl stress in uremia [14]. Furthermore, CML represents an important ligand for the AGE-receptor [15]. CEL, a structural anologue of CML, is formed from the reaction of lysine and methylglyoxal or in the course of lipid peroxidation during aging [16]. As it is known that, in addition to lysine, also arginine represents a major target for carbohydrate-induced protein modification, albumin samples derivatized on arginine (included to distiguish from CML and CEL which originate from modification of lysine) with methylglyoxal and glyoxal were submitted to evaluation as well. During reaction of arginine with glucose degradation products, imidazolinones are formed as predominant reaction product [17–19]. Recent reports indicate that the methylimidazolinone derived from methylglyoxal is accumulated as one of the AGEs in uremia [20].

The present study investigates the effect of these specific AGEs present in uremia on leukocyte function by analyzing free radical production, CD14 expression and DNA synthesis [5-bromo-2′-deoxyuridine (BrdU) incorporation] by calcitriol-differentiated HL-60 cells (monocyte/macrophage phenotype).

METHODS

Preparation of AGEs

Carboxymethylated albumin (CML albumin). Glyoxylic acid (3.5 mmol per g albumin) was added to a solution of bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, MO, USA) (20 mg/mL) in phosphate buffer (1/15 mol/L, pH 7.4), corresponding to a molar ratio between glyoxylic acid and lysine of 4:1. The pH was adjusted to 7.4 with 0.5 N NaOH, followed by addition of 8.8 mmol $NaBCNH_3$ per g albumin. After incubation for 20 hours at 40°C under constant stirring, the solution was extensively dialyzed against distilled water and freeze dried, resulting in a slightly yellow, crystalline powder. Content of CML was measured by gas chromatography-mass spectrometer (GC-MS) as described.

Carboxyethylated albumin (CEL albumin). Pyruvic acid (13 mmol per g albumin) was added under nitrogen to a solution of BSA (20 mg/mL) in phosphate buffer (1/15 mol/L, pH 7.4), corresponding to a molar ratio between pyruvic acid and lysine of 15:1. The pH was adjusted to 7.4 with 0.5 N NaOH, followed by addition of 39.6 mmol $NaBCNH_3$ per g albumin. After incubation for 20 hours at 40°C under constant stirring, this solution was extensively dialyzed against distilled water and freeze dried, equally resulting in a slightly yellow, crystalline powder. Content of CEL was measured by GC-MS as described below.

Arginine-modified albumin I and II (Arg I albumin and Arg II albumin). Respectively, 0.452 mmol glyoxal (for Arg I albumin) or methylglyoxal (for Arg II albumin) were added to a solution of BSA (20 mg/mL) in phosphate buffer (1/15 mol/L, pH 7.4). After incubation for 20 hours at 40°C under constant stirring, the solution was extensively dialyzed against distilled water and freeze dried, resulting in a yellow-brown, crystalline powder.

Evaluation of the prepared AGEs by chromatographic analysis

CML *albumin and* CEL *albumin.* CML and CEL were measured as the corresponding trifluoroacetylated methyl esters via GC-MS after acid hydrolysis. GC-MS was performed on a HP 6890 Plus GC system with HP 5973 mass selective detector, using a HP-5 phenylmethyl-siloxane capillary column from Agilent Technologies (Waldbronn, Germany). Protein samples were hydrolyzed for 23 hours at 110°C in the presence 6 N hydrochloric acid under a nitrogen atmosphere. After adding a suitable internal standard compound the hydrolysate was dried in vacuo, dissolved in 2 mL of 2 N methanolic HCl and kept at 65°C for 30 minutes. After this, the solution was dried in vacuo, dissolved in 500 μL of dichloromethane and 1 mL trifluoroacetic acid anhydride and kept at room temperature for 1 hour. After drying under nitrogen, the residue was dissolved in 300 μL of dichloromethane and submitted to GC-MS analysis. Conditions of GC and MS have been described elsewhere [13].

Arginine derivatization. Protein samples were hydrolyzed for 23 hours at 110°C in the presence 6 N hydrochloric acid under a nitrogen atmosphere. The hydrolysate was dried in vacuo and dissolved in 0.2 N sodium citrate buffer, pH 2.2. Amino acid analysis was performed on an Alpha Plus amino acid analyzer (LKB-Biochrom, Cambridge, UK), using a stainless steel column (150 × 4 mm) filled with ion-exchange resin, DC4A-spec sodium form (Benson, Reno, NV, USA).

Composition of elution buffers, ninhydrin reagent as well as running conditions was described elsewhere [21, 22].

Evaluation of biologic impact of AGEs

Cell line culture. HL-60 cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and were maintained as a continuous culture in RPMI 1640 medium supplemented with 2 mmol/L L-glutamine, 20% heat-inactivated fetal bovine serum (FBS) (Life Technologies, Merelbeke, Belgium) and 50 μg/mL gentamycin in a humidified atmosphere of 5% CO_2 in air at 37°C. Medium was refreshed every 3 to 4 days. Cell cultures were free of mycoplasma.

Experimental media. HL-60 cells were cultured, over a week period, in (*1*) medium alone, (*2*) medium containing unmodified serum albumin (albumin was run through the same modification process, but in the absence of the modifying compound) or AGE-modified albumin (CML albumin, CEL albumin, Arg I albumin, and Arg II albumin), (*3*) medium containing 10 nmol/L calcitriol to differentiate HL-60 toward the monocyte/macrophage phenotype [23], and (*4*) a combination of (2) and (3). The conditions (1) and (2) were included as control conditions. HL-60 has been proven to be an excellent model for studying the differentiation of leukocytes and their capacity to destroy infective agents. The monocyte/macrophage phenotype (after calcitriol induced differentiation) is of major importance in the process of atherogenesis. Cell viability at the moment of evaluation exceeded 90% as assessed by propidium iodide exclusion by flow cytometric analysis. The unmodified and modified serum albumin samples were used at a concentration of 1 mg protein/mL, resulting in a final concentration of 0.136 mg CML, 0.161 mg CEL, 0.050 mg Arg I or 0.066 mg Arg II per mL cell culture medium. AGE compounds were determined to contained between 0.2 and 0.7 IU/mL endotoxin (Endosafe® Endochrome-K™) (Charleston, SC, USA).

Evaluation methods for HL-60 cells.

Chemiluminescence production. Chemiluminescence production by HL-60 cells was determined after 1 week incubation in the different culture media and after readjustment of the cell count to 1×10^6 cells/mL in every sample. We added 500 μL of luminol solution (56 μmol/L) and 100 μL Hanks balanced salt solution (HBSS) or 100 μL phorbol 12-myristate 13-acetate solution (PMA) (1.5 μg/mL final concentration) to 50 μL of cell suspension. The test tube was immediately processed into a luminescence analyzer (Lumicon, Hamilton, Switzerland). The photon-counting value, which is indicated arbitrarily in relative light units (RLU), was registered with a counting interval set at 30 seconds and a total recycling time of 30 minutes. Chemiluminescence production was expressed as integrated chemiluminescence emission in counts/30 minutes, chemiluminescence peak in counts/min and slope.

Analysis of membrane-bound CD14 expression on HL-60 cells. Expression of CD14, a differentiation marker and receptor for lipopolysaccharide (LPS), was assessed by direct immunofluorescence after 3 days of culture. Fifty microliter cell suspension was incubated with Simultest™ Leucogate™ (Becton Dickinson, San Jose, CA, USA) at 4°C in the dark. Simultest™ Leucogate™ contains fluorescein isothiocyanate (FITC)-conjugated CD45 monoclonal antibodies (anti-HLe-1) and phycoerythrin (PE)-conjugated CD14 monoclonal antibodies (Leu™-M3). After washing procedures, the cells were submitted to flow cytometric analysis (FACScan®) (Becton Dickinson). Fluorescence was standardized by microbeads (Calibrite™ particles) (Becton Dickinson) with amplification and voltage kept constant throughout the procedure. Analysis was performed on 10,000 events (detector threshold forward scatter-height (FSC-H):200, parameter FSC-H:1.00). The cell population was gated according to forward and right-angled light scatter. Background binding was estimated by isotype-matched negative control antibodies (Simultest™ Control) (Becton Dickinson).

BrdU-DNA synthesis. At day 7 of incubation, cell proliferation by the measurement of the incorporation of BrdU into the DNA was evaluated. Anti-BrdU (Becton Dickinson) was used in flow cytometric analysis to identify cells that synthesized DNA during exposure to BrdU (Sigma Chemical Co).

Cells were incubated for 30 minutes with BrdU (10 μmol/L) in the CO_2 incubator at 37°C. After two washings with 1% BSA/phosphate-buffered saline (PBS), the pellet was resuspended in 200 μL of PBS on ice and to fix the cells, this suspension was then slowly added to 5 mL of 70% ethanol (−20°C) followed by a 30-minute incubation period on ice. After centrifugation and aspiration of the supernatant, 1 mL 2 N HCl/0.5% Triton X-100 was slowly added to the pellet followed by a 30-minute incubation period at room temperature to denature the DNA. After centrifugation, 1 mL of 0.1 mol/L $Na_2B_4O_4.10\,H_2O$, pH 8.5, was added to the pellet to neutralize the acid, followed by a second centrifugation step. Cell concentration was adjusted with 0.5% Tween/1.0% BSA/PBS to achieve 10^6 cells/test. Cells were incubated with FITC-conjugated anti-BrdU for 30 minutes and washed once in 1 mL 0.5% Tween/1.0% BSA/PBS, and finally resuspended in PBS containing 5 μg/mL propidium iodide before analysis by a FACScan® (Becton Dickinson).

Statistical analysis

Data are expressed as mean ± SD ($N = 6$). Statistical analysis was performed using one-way repeated measures

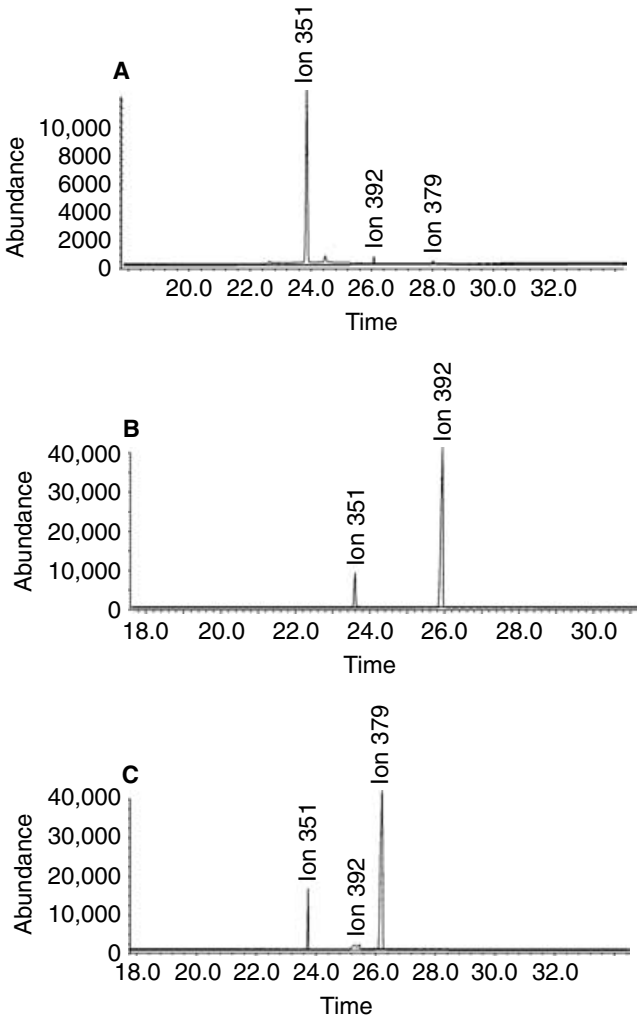

Fig. 1. Gas chromatography-mass spectrometry (GC-MS) chromatograms of native bovine serum albumin (*A*) compared to the carboxymethylated (*B*) and carboxyethylated (*C*) form. N-δ-carboxymethylornithine (m/z 351), a naturally not occurring homologue of N-ε-carboxymethyllysine, was added before sample preparation and used as an internal standard. N-ε-carboxymethyllysine (m/z 392) (B) and N-ε-carboxyethyllysine (m/z 379) (C) are both products of specific synthesis and were selectively enriched in the corresponding protein.

of analysis of variance (ANOVA) followed by a paired Wilcoxon signed rank test in case of significant ANOVA. A P value of less than 0.05 was considered significant.

RESULTS

Evaluation of the prepared AGEs by chromatographic analysis

For the synthesis of albumin modified with CML and CEL, reductive alkylation of the protein in the presence of glyoxylic acid or pyruvic acid, respectively, was performed. This procedure offers the possibility to selectively modify lysine residues avoiding other typical Maillard reaction products as illustrated in Figure 1, where Figure 1A shows the GC-MS chromatograms of native BSA in comparison to the carboxymethylated (Fig. 1B) and carboxyethylated (Fig. 1C) form.

The amount of the lysine derivatives formed under these conditions was 136 mg CML per g of protein and 161 mg CEL per g of protein, respectively, as measured via GC-MS after acid hydrolysis. These concentrations correspond to a lysine modification within the albumin preparations of 74.8% (by CML) or 83.9% (by CEL). For the albumin samples incubated with glyoxal or methylglyoxal, the derivatization of arginine was quantified after acid hydrolysis on the amino acid analyzer as 67.1% for glyoxal-modified albumin (Arg I albumin) and 83.1% for methylglyoxal-modified albumin (Arg II albumin), respectively, 50.3 and 65.7 mg/g protein. For both preparations, arginine derivatization can be explained by the formation of imidazolinones or chemically related compounds [17–19]. Compared to arginine, derivatization of lysine in Arg I albumin and Arg II albumin was negligible.

Table 1. Effect of advanced glycation end-product (AGE) compounds on the characteristics of the luminol-amplified chemiluminescence production of phorbol 12-myristate 13-acetate (PMA)-stimulated HL-60 cells

	Medium	AGE	Calcitriol	Calcitriol + AGE
Arg I albumin				
Integral	9.8 ± 6.0	13.2 ± 13.8	46.4 ± 18.8[a]	66.1 ± 32.6[a,b]
Peak	6.7 ± 3.7	5.1 ± 3.4	27.8 ± 6.6[a]	43.2 ± 17.9[a,b]
Slope	1.4 ± 1.7	0.9 ± 0.8	6.3 ± 1.9[a]	8.5 ± 3.8[a]
Arg II albumin				
Integral	12.2 ± 7.5	8.1 ± 3.5	55.6 ± 37.1[a]	61.9 ± 38.6[a]
Peak	8.0 ± 3.7	6.3 ± 3.5	37.6 ± 27.4[a]	44.4 ± 27.8[a]
Slope	1.5 ± 0.8	1.7 ± 1.2	8.1 ± 5.3[a]	9.9 ± 9.8[a]
CEL albumin				
Integral	8.2 ± 7.4	4.9 ± 1.8	44.1 ± 18.5[a]	64.7 ± 28.1[a,b]
Peak	4.2 ± 1.6	4.3 ± 2.4	46.5 ± 18.9[a]	57.4 ± 25.8[a,b]
Slope	2.2 ± 1.4	0.6 ± 0.7	12.9 ± 7.7[a]	14.4 ± 9.4[a]
CML albumin				
Integral	2.7 ± 1.3	2.2 ± 0.6	41.9 ± 25.5[a]	60.9 ± 5.5[a,c]
Peak	2.8 ± 1.8	2.3 ± 1.4	27.0 ± 17.3[a]	62.3 ± 63.9[a,b]
Slope	0.7 ± 1.4	1.3 ± 2.4	6.8 ± 7.4[a]	8.7 ± 9.7[a]

Abbreviations are: CML, N-ε-carboxymethyllysine; CEL, N-ε-carboxyethyllysine; integral (counts 10^3/30 min); peak (cpm 10^3); slope (10^3); mean ± SD.
[a] $P < 0.05$ versus medium; [b] $P < 0.05$ and [c] $P = 0.0625$ versus calcitriol ($N = 6$).

Effect of the different AGE compounds on production of free radicals (chemiluminescence production)

The presence of serum albumin per se in the culture medium had no effect on the chemiluminescence production of HL-60 cells cultured in the absence and the presence of calcitriol (data not shown). Baseline experiments, without PMA stimulation, showed no effect of the AGE compounds, neither in medium alone, nor in calcitriol-supplemented medium (data not shown). The study of the effect of AGE compounds on the PMA-stimulated chemiluminescence production of undifferentiated HL-60 cells, applied as control experiments, showed no effect (Table 1). As expected, the addition of calcitriol

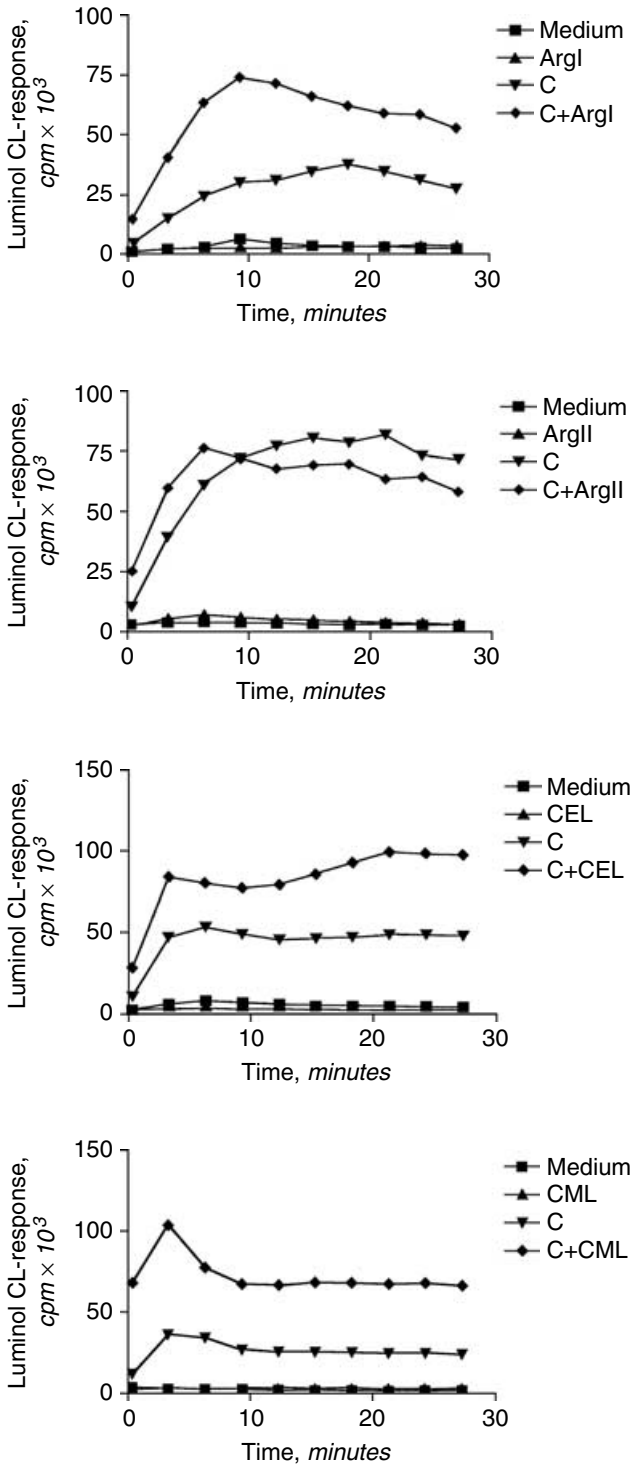

Fig. 2. Representative time curves of luminol-dependent chemiluminescence (CL) assay from phorbol 12-myristate 13-acetate (PMA)-stimulated HL-60 cells cultured in medium, in medium supplemented with Arg I albumin, Arg II albumin, N-ε-carboxyethyllysine (CEL) albumin, or N-ε-carboxymethyllysine (CML) albumin both in the absence and the presence of calcitriol (C). Calcitriol-treated cells are to be considered as the equivalent of monocyte/macrophage cells. In parallel to the average results illustrated in Table 1, there is a marked increase in response of the calcitriol-treated cells in the presence of Arg I albumin, CEL albumin, and CML albumin. The impact of Arg II albumin is neutral.

Table 2. Effect of different advanced glycation end-product (AGE) compounds on the expression of membrane bound CD14 on calcitriol-differentiated HL-60 cells

	% CD14-positive cells		Mean fluorescence intensity	
	Calcitriol	Calcitriol + AGE	Calcitriol	Calcitriol + AGE
Arg I albumin	58 ± 3	62 ± 4[a]	768 ± 143	890 ± 150[a]
Arg II albumin	79 ± 3	80 ± 3	1160 ± 149	1177 ± 208
CEL albumin	71 ± 11	77 ± 9[a]	1695 ± 796	2075 ± 1044[a]
CML albumin	55 ± 9	69 ± 7[a]	647 ± 63	716 ± 69[a]

Abbreviations are: CML, N-ε-carboxymethyllysine; CEL, N-ε-carboxyethyllysine. Mean ± SD.
[a] $P < 0.05$ versus calcitriol ($N = 6$).

to the culture medium induced cell differentiation (toward the monocyte/macrophage phenotype) resulting in a marked increase in free radical production in response to PMA. The presence of Arg I albumin, CEL albumin, and CML albumin in the culture medium enhanced the PMA-stimulated chemiluminescence production of the calcitriol differentiated HL-60 cells (Arg I albumin and CEL albumin) ($P < 0.05$ versus calcitriol) and (CML albumin) ($P = 0.0625$ versus calcitriol) (integral and peak values) (Table 1) (Fig. 2). Even if no significant changes were found for the slope values, the same trend was maintained as for the other parameters. The presence of Arg II albumin in the calcitriol containing culture medium did not influence the chemiluminescence production.

Effect of the different AGE compounds on the expression of membrane-bound CD14

Undifferentiated HL-60 cells in culture do not express CD14 on their surface membrane, CD14 expression is induced when cells are cultured in the presence of calcitriol ($P < 0.05$). The presence of serum albumin per se in the culture medium did not influence the CD14 expression (data not shown). Table 2 illustrates that when these calcitriol-differentiated HL-60 cells were cultured in the presence of Arg I albumin, CEL albumin, and CML albumin the percentage of CD14-positive cells as well as the amount of CD14 expression per cell was significantly enhanced ($P < 0.05$).

Effect of the different AGE compounds on DNA synthesis (BrdU incorporation)

The presence of serum albumin per se in the culture medium had no effect on the DNA synthesis of HL-60 cells cultured in the absence or presence of calcitriol (data not shown). None of the tested AGE compounds had an effect on the undifferentiated HL-60 cells (Table 3) (Fig. 3).

When HL-60 cells were cultured in the presence of calcitriol resulting in a differentiation toward the monocyte/macrophage phenotype, the percentage of HL-60

Table 3. Effect of different advanced glycation end-product (AGE) compounds on DNA synthesis [5-bromo-2′-deoxyuridine (BrdU) incorporation] of HL-60 cells

	Medium	AGE	Calcitriol	Calcitriol + AGE
Arg I albumin	36.5 ± 4.6	33.4 ± 6.7	27.7 ± 9.7[a]	34.3 ± 3.6[c]
Arg II albumin	36.2 ± 4.5	33.9 ± 6.9	33.0 ± 4.1[b]	32.0 ± 2.5
CEL albumin	30.5 ± 2.8	29.68 ± 2.0	23.3 ± 2.1[a]	22.1 ± 2.4
CML albumin	41.4 ± 3.2	42.1 ± 2.3	34.2 ± 3.3[a]	35.1 ± 0.8

Abbreviations are: CML, N-ε-carboxymethyllysine; CEL, N-ε-carboxyethyllysine; BrdU incorporation (% BrdU-positive cells).
[a] $P < 0.05$; [b] $P = 0.0625$ versus medium; [c] $P < 0.05$ versus calcitriol ($N = 6$).

cells incorporating BrdU (synthesizing DNA) was significantly decreased (overall from 36.5 ± 4.6 to 27.7 ± 9.7% BrdU-positive cells) ($P < 0.05$, $N = 6$), confirming the known antiproliferative effect of calcitriol (Fig. 3). The presence of Arg I albumin in the calcitriol-containing culture medium again induced more DNA synthesis (34.3 ± 3.6% BrdU-positive cells) ($P < 0.05$) (Fig. 3). No such effect was observed for CML albumin, CEL albumin, or Arg II albumin (Table 3).

DISCUSSION

In this study, the biologic effects of albumin containing genuine AGE structures (CML albumin, CEL albumin, Arg I albumin, and Arg II albumin, which are known to be retained in uremia, were studied, with specific emphasis on leukocyte function. Arg I albumin, CML albumin and CEL albumin were found to enhance free radical production and CD14 expression of cells of the monocyte/macrophage phenotype. In addition, Arg I albumin also increased the DNA synthesis.

AGEs result from irreversible modifications of amino acids, proteins, or peptides by carbohydrates and other metabolites. Oxidative and carbonyl stress have been pointed out as responsible events for their generation in uremia [7, 24, 25]. AGEs have been reported to be the result of inflammation [26] but at the same time they have been shown, in vitro, to cause inflammation [27, 28]. In a recent study by Bernheim et al [27], the superoxide production of leukocytes incubated in the presence of a mix of AGE compounds was activated at baseline but was attenuated upon stimulation. In this way, leukocyte dysfunction may at baseline contribute to the inflammatory status of the uremic patient while the attenuated response upon stimulation may have its implications in the increased susceptibility to infection.

In most in vitro studies, however, AGE-modified proteins were prepared, as mixed modified samples by simple incubation of the protein or albumin solution in the presence of glucose, generally without detailed chemical characterization of the formed amino acid derivatives and without the possibility to analyze individual compounds as they are retained in uremia. In this context, it remains a matter of debate whether the latter mixes of AGEs are representative for the AGEs retained in vivo [29].

For the time being, it has not yet been decided which of the individual AGE compounds defined in uremia exert toxic biologic effects and actually virtually no individual AGEs have been shown to be bioactive [30]. In the present study, the effect of chemically well-defined AGE compounds, the protein-bound lysine derivatives, CML albumin and CEL albumin, as well as the protein-bound arginine modifications, Arg I albumin and Arg II albumin, induced by glyoxal and methylglyoxal, on leukocyte functions like cell differentiation and cell proliferation, was evaluated. To the best of our knowledge, these specific compounds, characteristic for uremic AGE retention, have not previously been submitted to an evaluation of their separate biologic activity. Since, in the present study, we focused each time on isolated AGE compounds and not on heterogenously glycated AGEs (containing several compounds together) we aimed at higher concentrations, to be able to link possible effects to specific stuctures. It is of note that, in contrast to CML, up to now no quantitative data are available for the individual arginine derivatives [31]. In the past, these compounds have been detected qualitatively using immunologic methods. Therefore, it is not yet possible to compare the used concentrations with levels in uremia.

Calcitriol is known to differentiate HL-60 cells toward the monocyte/macrophage phenotype. The latter leukocyte phenotype is of major importance in the process of atherogenesis. The separate AGE compounds, Arg I albumin, CEL albumin, and CML albumin, significantly increased the PMA-stimulated chemiluminescence production and the CD14 expression on calcitriol differentiated HL-60 cells compared to unmodified serum albumin. An enhanced production of free radical species in the presence of specific AGE compounds could contribute to the inflammatory status of patients with ESRD. In addition, an overexpression of the differentiation antigen which is a receptor for LPS could result in an overexpression of proinflammatory cytokines which may also induce or contribute to an inflammatory status [32]. This activation could be of relevance in connection with the known baseline inflammatory status of end-stage renal failure patients [33]. It is of note, however, that a recent study reported an inverse correlation between the concentration of certain AGEs and the development of cardiovascular disease in hemodialysis patients [34]. It should be considered, however, that the AGEs evaluated in the above study might be not the ones affecting the immune or cardiovascular system.

Our data on cell proliferation showed that only one of the tested AGE compounds, Arg I, enhanced the DNA synthesis of calcitriol-differentiated HL-60 cells. An increase in cell proliferation was also reported when

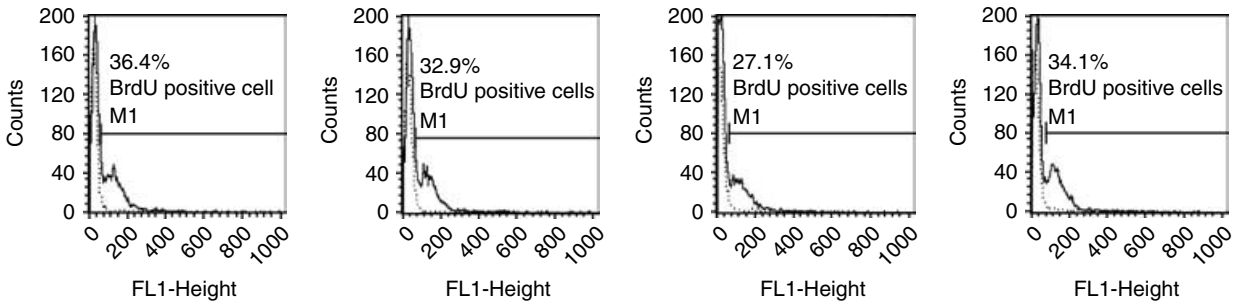

Fig. 3. A representative experiment of the evaluation of 5-bromo-2′-deoxyuridine (BrdU) incorporation into DNA (DNA synthesis) of HL-60 cells cultured in medium, in medium supplemented with Arg I albumin both in the absence and the presence of calcitriol [(C) and (C + Arg I albumin)]. A marker (M1) is set on the isotype control sample to exclude aspecific staining. The percentage of BrdU-positive cells is noted.

osteoblast-like cells were cultured in the presence of AGE, although this effect was time-dependent and cell proliferation was found to be depressed again after a longer (days) incubation period [35]. Glycated serum albumin has also been reported to stimulate cell proliferation of vascular smooth muscle cells [36]. In contrast, AGEs impair cell proliferation of human mesangial cells and renal tubular cells (LLC-PK1) [37, 38]. These data point to the fact that the effect of AGEs is, very much, cell type dependent.

CONCLUSION

The present study points out, for the first time, that Arg I albumin, CML albumin, and CEL albumin, which are specific AGE compounds known to be elevated and present in uremia, exert a biologic activity on leukocytes from the monocyte/macrophage phenotype which could very likely contribute to the progression of atherosclerosis.

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