Advanced Glycation End Products Stimulate Tumor Necrosis Factor-Alpha and Interleukin-1 Beta Secretion by Peritoneal Macrophages in Patients on Continuous Ambulatory Peritoneal Dialysis

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Abstract

Background: Advanced glycation end products, formed by the non-enzymatic glycation of proteins with reducing sugars, are thought to play a pathogenetic role in the vascular complications of diabetes, uremia and atherosclerosis. β2-microglobulin is a major constituent of amyloid fibrils in dialysis-related amyloidosis. AGE1-modified β2m has been found in amyloid deposits of long-term hemodialysis patients. AGE-modified β2m has also been shown to enhance chemotaxis and increase tumor necrosis factor-alpha and interleukin-1 beta secretion by circulating and tissue monocytes/macrophages.

Objectives: To investigate the effect of AGE-modified β2m and AGE-human serum albumin on TNF-α and IL-1β secretion by human peritoneal macrophages derived from patients on continuous ambulatory peritoneal dialysis.

Methods: Human PMØ were isolated from peritoneal dialysis effluent of stable CAPD patients and were incubated for 24 hours with AGE-modified β2m, β2m, AGE-HSA, HSA or lipopolysaccharide. TNF-α or IL-1β secretion was measured by enzyme-linked immunosorbent assay in cell-free culture supernatants.

Results: Both AGE-modified β2m and AGE-HSA significantly increased TNF-α and IL-1β secretion by human PMØ in a dose-dependent manner (50–200 µg/ml). In contrast, β2m or HSA had no such stimulatory effect on TNF-α secretion but had a small significant increase in IL-1β secretion.

Conclusions: AGE-modified β2m promotes in vitro TNF-α and IL-1β secretion by human PMØ of CAPD patients. Activation of these macrophages by AGE-modified β2m may be a contributory factor to the morphologic changes and altered permeability of the peritoneal membrane in long-term CAPD.

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Advanced glycation end products are a heterogeneous group of compounds produced by the non-enzymatic Maillard reaction between protein and sugars [1,2]. Once formed, AGEs are, by nature, irreversible. They are characterized by a propensity to cross-link proteins and may trigger complex cellular interactions through a specific cell membrane receptor system, whose presence on macrophages has previously been documented [2,3]. Levels of AGEs are increased in diabetes, renal failure and natural aging [2]. AGEs have been implicated in the pathogenesis of the complications of diabetes mellitus (nephropathy, retinopathy, neuropathy), renal failure and aging, and in the genesis of atherosclerotic vasculopathy [2,4,5].

Dialysis-related amyloidosis is a disabling disease that affects patients on long-term dialysis [6]. Amyloid deposits are mainly located in joint structures, especially in periarticular bones, leading to a progressive destructive arthropathy [6]. β2-microglobulin and, in particular, its acidic isoform is the major constituent of the amyloid fibrils in dialysis-related amyloidosis [7]. Recent studies have demonstrated that β2m isolated from dialysis-related amyloidosis tissue is modified by AGEs [8,9]. Proteins with long half-lives, such as amyloid fibril subunit proteins, are more susceptible to AGE modification [8]. AGE-modified β2m has been demonstrated in the sera and urine of these patients but not in those of healthy individuals with normal renal function [8]. AGE-modified β2m, but not normal β2m, has been shown to enhance chemotaxis and increase the secretion of tumor necrosis factor-alpha and interleukins 1 beta and 6 from circulating and/or tissue monocytes/macrophages [10,11]. A possible contributing role of AGE-modified β2m in the formation of dialysis-related amyloidosis has therefore been suggested.

In continuous ambulatory peritoneal dialysis, the peritoneal membrane is continuously exposed to extremely high glucose concentrations. Local generation of AGEs and their deposition in the peritoneal capillary wall have been described [12,13]. Human peritoneal macrophages from CAPD patients with bacterial peritonitis are capable of upgrading their production of β2m. AGE-modified β2m is, in all probability, present in the peritoneal effluent. The structural changes induced by AGEs are thought to play a role in the altered permeability characteristics of the peritoneal membrane in patients on long-term CAPD [14].

In this study we evaluated the effect of AGE-modified β2m on TNF-α and IL-1β secretion by human peritoneal macrophages obtained from patients on CAPD, comparing it to that found with AGE-human serum albumin, an accepted AGE-modified control protein.

AGE = advanced glycation end products
β2m = β2-microglobulin
TNF-α = tumor necrosis factor-alpha
IL-1β = interleukin-1 beta
PMØ = peritoneal macrophages
CAPD = continuous ambulatory peritoneal dialysis
HSA = human serum albumin

Patients and Methods

Patients

Human PMØ were isolated from the peritoneal dialysis effluent of 16 CAPD patients (mean age 67.2 ± 13.9 years, mean duration of peritoneal dialysis 12.7 ± 6.1 months) who had been without peritonitis for at least 6 months prior to the investigation. They were non-diabetic patients using standard Dialine® (TEVA-Medical, Israel) peritoneal dialysis solutions with low pH (5.2–5.6) and glucose (1.5–4.25 g/dl) as the osmotic agent.

PMØ Isolation and stimulation

The complete effluent from an overnight dwell was centrifuged at 450 *g* at 4°C for 25 minutes. Cells were washed three times in phosphate-buffered saline without Ca^{++} and Mg^{++} and resuspended in RPMI-1640 medium supplemented with 5% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Tissue culture reagents were purchased from Biological Industries (Beit HaEmek, Israel). Then, 2 x 10^6 cells were seeded onto six-well microplates and incubated at 37°C at an atmosphere of 5% CO_2 in air. An hour later non-adherent cells were rinsed away by warm medium. More than 95% of adherent cells were consistently found to be PMØ, morphologically characterized by non-specific esterase staining (Sigma, Israel). Viability was assessed by trypan blue exclusion and found to be greater than 90%.

The PMØ were incubated with increasing concentrations of AGE-modified β2m, β2m, AGE-HSA, HSA (0–200 µg/ml), LPS (2 µg/ml: E*scherichia coli* 055:B5, Sigma, Israel) as positive control and medium. After 24 hours, cell-free supernatants were collected and stored at -20°C until the measurement of TNF-α or IL-1β.

Cytokine immunoassay

TNF-α and IL-1β in macrophage culture supernatants were measured using a commercial enzyme-linked immunosorbent assay kit (Endogen, USA) according to the manufacturer's instructions. The sensitivity of the assay was 2 pg/ml for TNF-α and 1 pg/ml for IL-1β. TNF-α and IL-1β levels are given as pg/10^6 PMØ after correction of cell number based on the total number of adherent cells.

Preparation of AGE-HSA and AGE-modified β2m

AGE-HSA and AGE-modified β2m were prepared as previously described [8]. Briefly, HSA (0.75 mmol/L, Sigma, Israel) or β2m (0.17 mmol/L, ICN, USA) were dissolved with 0.5 mol/L (for AGE-HSA preparation) or 0.1 mol/L (for AGE-modified β2m) of glucose (Riedel-de Haen, Germany) in 100 mmol/L sodium phosphate buffer (pH 7.4) containing 200 U/ml of penicillin, 200 µg/ml streptomycin, 80 µg/ml gentamycin, and 1.5 mmol/L phenylmethylsulfonylfluoride under sterile conditions. Sterilization was initially performed with 0.22 µm pore size filters after which the solution was incubated for 60 days in the dark at 37°C. After incubation the solution was dialyzed overnight against PBS. The HSA or β2m controls were subjected to the same procedure (except for the presence of glucose in the incubating solution) as that used for AGEs. Final products contained less than 0.2 ng/ml (corresponding to < 0.96 EU/ml) of endotoxin (E-Toxate, Sigma). The concentration of AGE-HSA, AGE-modified β2m, HSA and β2m were determined by the method of Bradford.

Measurement of AGEs

The AGE-HSA and AGE-modified β2m fluorescence was measured with excitation/emission 370/440 nm on the spetrofluorophotometer RF-1501 (Shimadzu, Japan). AGEs were also evaluated by ELISA using anti AGE monoclonal antibody (clone 6D, Wako Chemicals, USA).

Statistical analysis

The results are expressed as mean ± SE. Multiple comparisons between different groups were performed with ANOVA. *P* values less than 0.05 were considered significant.

Results

Effect of AGE-modified β2m and AGE-HSA on TNF-α secretion by human PMØ, *in vitro*

With increasing concentration of AGE-modified β2m (50–200 µg/ml), a linear increase in TNF-α secretion is seen, as compared to β2m or to the basal condition reaching significance at concentrations of 100 and 200 µg/ml. A similar response to TNF-α secretion was seen by AGE-HSA. The control groups of β2m and HSA (50–200 µg/ml) had no stimulatory effect on TNF-α secretion. LPS (2 µg/ml), a positive control, significantly increased TNF-α secretion by PMØ ($P < 0.001$) [Table 1].

Effect of AGE-modified β2m and AGE-HSA on IL-1β secretion by human PMØ, *in vitro*

AGE-modified β2m, β2m, AGE-HSA and HSA showed a significant increase in IL-1β secretion by human PMØ in a dose-dependent manner. There was a significant stimulating action

Table 1. Effect of AGE-modified β2m and AGE-HSA on TNF-α secretion by human PMØ, *in vitro*[a]

	TNF-α (pg/10^6 cells)				
Concentration (µg/ml)	AGE–modified β2m	β2m	AGE-HSA	HSA	LPS
0	15.6 ± 9.6		15.6 ± 6.5		
50	72.0 ± 21.2	10.4 ± 4.3	45.5 ± 15.6	9.3 ± 5.9	
100	259.3 ± 63[b,c]	5.3 ± 2.2	187.0 ± 45.1[b,d]	10.2 ± 6.0	
200	401.7 ± 42.1[b,c]	9.2 ± 4.6	302.5 ± 77.3 [b,d]	8.4 ± 4.9	
2 (LPS)					2396 ± 102.5[b]

[a] Human PMØ were incubated with AGE-modified β2m, β2m, AGE-HSA, HSA (0–200 µg/ml) or LPS (2 µg/ml). After 24 hours of incubation, cell-free supernatants were collected and tested for TNF-α. The data present a mean ± SE of five different experiments.

[b] $P < 0.001$ vs. 0

[c] $P < 0.001$ vs. β2m (100 or 200 µg/ml)

[d] $P < 0.004$ vs. HSA (100 or 200 µg/ml)

LPS = lipopolysaccharide
PBS = phosphate-buffered saline
ELISA = enzyme-linked immunosorbent assay

Table 2. Effect of AGE-modified β2m and AGE-HSA on IL-β secretion by human PMØ, in vitro[a]

Concentration (μg/ml)	IL-1β (pg/10^6 cells)				
	AGE--modified β2m	β2m	AGE-HSA	HSA	LPS
0	9.9 ± 4.5		8.8 ± 5.9		
50	466.3 ± 309.3	33.5 ± 13.7	207.8 ± 79.6	18.4 ± 11.5	
100	1210.8 ± 271.9[b,c]	159.0 ± 32.1[b,e]	870.3 ± 216.5 [b,d]	101.0 ± 18.2 [b,f]	
200	1564.5 ± 560.9[b,c]	260.3 ± 80.9[b,e]	1380.5 ± 472.0 [b,d]	216.5 ± 40.9 [b,f]	
2 (LPS)					2916 ± 105[b]

[a] Human PMØ were incubated with AGE-modified β2m, β2m, AGE-HSA, HSA (0–200 μg/ml) or LPS 2 μg/ml). After 24 hours of incubation, cell-free supernatants were collected and tested for IL-β. The data present a mean ± SE of five different experiments.

[b] $P < 0.007$ vs. 0

[c] $P < 0.03$ vs. β2m (100 or 200 μg/ml)

[d] $P < 0.01$ vs. HSA (100 or 200 μg/ml)

[e] $P < 0.01$ vs. β2m (50 μg/ml)

[f] $P < 0.01$ vs. HSA (50 μg/ml)

of AGE-modified β2m and AGE-HSA when compared with β2m or HSA or the basal condition. LPS (2 μg/ml), a positive control, significantly increased IL-1β secretion by PMØ ($P < 0.007$) [Table 2].

Discussion

It has been firmly established that β2-microglobulin is the major constituent of the amyloid fibrils found in dialysis-related amyloidosis [6,7]. Biochemical modifications of the β2m molecules may facilitate its deposition within amyloid fibrils. Among these is AGE-modified β2m. Miyata et al. [8] have shown that the acidic isoform of β2m, produced on incubation of normal β2m with glucose, possesses properties characteristic of AGEs and is a dominant constituent of amyloid deposits in dialysis-related amyloidosis. This group then extended their studies, demonstrating that acidic β2m is capable of inducing monocyte chemotaxis and macrophage secretion of bone-resorbing cytokines, namely TNF-α, IL-1β and IL-6 [10,11,15]. These findings implicated AGEs in the pathogenesis of dialysis-related amyloidosis.

Glomerular filtration and eventual degradation by the renal tubules constitute the main route of disposal of β2m. Serum levels are inversely correlated to residual renal function. On standard CAPD therapy (four exchanges/day), peritoneal clearance of β2m has been reported to range between 0.99 and 1.1 ml/min [16,17]. In CAPD, β2m diffuses into the peritoneal cavity. It has also been shown, *in vitro*, that PMØ from CAPD patients with bacterial peritonitis are capable of upgrading their production of β2m [17]. β2m is, therefore, a constant inhabitant within the peritoneal dialysis effluent. In patients maintained on CAPD, the peritoneal membrane as well as the dialysis effluent is continuously exposed to very high glucose concentrations, a milieu conducive to AGE formation [12,13]. AGEs have been shown to be present in the peritoneal effluent and in the peritoneal capillary wall [12–14]. In concert with the formation of other AGE compounds, AGE-modified β2m is probably also present in the peritoneal dialysis effluent.

The present work showed an increase in the secretion of TNF-α and IL-1β by PMØ induced by both AGE-modified β2m and AGE-HSA. The non-AGE-modified counterpart proteins β2m and HSA also increased IL-1β secretion slightly. The stimulatory effect of AGE-modified β2m and AGE-HSA was dose-dependent. However, there was no significant difference in the secretory response between the two AGE proteins. These data suggest that it may be the AGE modification itself rather than the specific protein that is responsible for the induction of cytokine production. Our results confirm the data reported by Miyata et al. regarding the stimulatory effect of AGEs on monocytes [10,11,15]. Our study is unique in that it was conducted on peritoneal macrophages obtained from CAPD patients, whereas the aforementioned studies involved circulating and/or tissue monocytes/macrophages from hemodialysis patients [10,11,15].

In the basal state, human PMØ from CAPD patients demonstrate a decreased secretory response to immune stimuli [18]. However, *in vitro*, we and others have shown that these PMØ are capable of increasing cytokine secretion and are, in fact, in a state of chronic activation [13,19,20].

The quantity of AGEs has been shown to correlate with the progression of peritoneal interstitial fibrosis and vascular neoangiogenesis and sclerosis [21]. Nε-carboxymethyllysine, pentosidine and imadozolone are well-characterized AGEs that have been detected in the peritoneal effluent and peritoneal membrane of long-term peritoneal dialysis patients [14,21,22], as well as in amyloid deposits of long-term hemodialysis patients. The concentration of AGE-modified β2m in the peritoneal dialysis effluent has never been determined, but recently Cocklin et al. [23] described a new method for the identification and localization of AGE-modified β2m in human serum, namely, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. The use of this procedure will undoubtedly aid in the analysis of different forms of peritoneal AGEs.

The concentrations of AGEs in our experimental system were comparable to those employed by other investigators [10,15]. TNF-α levels after stimulation by AGEs were similar to those found post-stimulation of cultured CAPD peritoneal macrophages [24]. The increase in TNF-α and IL-1β secretion by PMØ may activate vascular smooth muscle cells to synthesize extracellular matrix components. TNF-α and IL-1β have also been shown to stimulate mesothelial cells, thereby altering the regulation of the cytokine network [25]. The end result will be neoangiogenesis and eventual fibrosis of the peritoneal membrane. Analogous to the proposed pathogenic role of AGE-modified β2m in the formation of dialysis-related amyloidosis, AGE-modified β2m-induced cytokine production should therefore be considered a potential causative factor of morphologic changes, increased permeability of the peritoneal membrane, and ultra-filtration failure in long-term CAPD patients.

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