Interaction between β2-microglobulin and advanced glycation end products in the development of dialysis related-amyloidosis

FAN FAN HOU, GLENN M. CHERTOW, JONATHAN KAY, JOSHUA BOYCE, J. MICHAEL LAZARUS, JAMES A. BRAATZ, and WILLIAM F. OWEN, JR.

Department of Medicine, Renal Division, Division of Immunology and Rheumatology, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts, USA; Department of Nephrology, Nanfang Hospital, Guangzhou, People's Republic of China; Section of Rheumatology, Lahey Hitchcock Medical Center, Burlington, Massachusetts, and W. R. Grace & Co.-Conn Washington Research Center, Columbia, Maryland, USA

Interaction between β2-microglobulin and advanced glycation end products in the development of dialysis related-amyloidosis. Dialysis related amyloidosis (DRA) is a progressive debilitating complication of long-term dialysis. β2-microglobulin (β2m) amyloid deposition occurs preferentially in older patients and initially is located in collagen-rich osteo-articular tissues. Since an age-dependent increase in the formation of advanced glycation end products (AGE) has been observed in collagen-containing structures, we hypothesized that AGE-modified β2m in the amyloid of DRA may be formed locally in osteo-articular structures as a subsequent event of its binding to collagen-AGE. Based on this hypothesis, we investigated the binding between β2m and AGE-modified collagen (collagen-AGE) in vitro. Significantly larger amounts of human β2m were bound to types I to IV of immobilized collagen-AGE than to unmodified collagens (P < 0.0001). The quantity of β2m bound to collagen-AGE was dependent on the concentrations of both β2m and of AGE contained in collagen (P < 0.01). Unmodified β2m was more avidly bound to collagen-AGE or collagen in comparison to AGE-modified β2m (P < 0.0001). β2m bound to collagen-AGE could be modified further by nonenzymatic glycosylation during three weeks of incubation with physiologic concentrations of glucose. Similar processes in vivo may be important in the pathobiology of DRA.

Dialysis-related amyloidosis (DRA) is a serious, often incapacitating complication for older patients undergoing long-term dialysis [1–3]. Initially, amyloid deposits are mainly located in osteo-articular tissue, leading to carpal tunnel syndrome, destructive arthropathy, and subchondral bone erosions and cysts [4–6]. Later in the course of the disease, deposition extends to the visera and vasculature [7]. Recent biochemical and immunohistological studies have demonstrated that β2-microglobulin (β2m) is a major constituent of the amyloid fibrils in DRA [8, 9]. β2m, a protein with a molecular weight of 11800 daltons, is produced by all nucleated cells and is normally eliminated by the kidney [10]. Thus, the β2m level is markedly elevated in persons with chronic renal failure as compared with healthy individuals [10]. However, no correlation between the serum or joint concentrations of β2m and the occurrence of DRA has been observed [11]. Furthermore, little is known about the mechanism for the preferential recruitment of β2m to osteo-articular structures.

Clinical observations have shown that the incidence and prevalence of DRA increase with age [2, 3]. Osteo-articular tissue, which is rich in collagen, is the most common site of deposition of β2m amyloid [1]. With advancing age, a linear increase in advanced glycation end product (AGE) accumulation in collagen has been demonstrated [12]. Therefore, we hypothesized that there is an affinity between β2m and AGE-modified collagen that might promote the preferential accumulation of β2m amyloid deposits in older persons with chronic renal failure.

Methods

Preparation of AGE-modified proteins

AGE-modified β2m and collagens were prepared in vitro as previously described [13]. Briefly, 1.75 mg/ml of purified normal human β2m (Cortex Biochem, San Leandro, CA, USA) or 0.45 mg/ml of purified type I (from calf skin; Sigma, St. Louis, MO, USA), type II (from bovine tracheal cartilage; Sigma), type III (from human placenta; Sigma), and type IV (from human placenta; Sigma) collagen were incubated separately at 37°C for 30 days with 100 mM D-glucose in 100 mM phosphate buffer containing 200 U/ml penicillin, 70 µg/ml gentamicin, and 1.5 mM PMSF. Samples incubated in an identical manner in the absence of glucose were used as controls. For some experiments, unincubated β2m or collagen were used as additional controls. After incubation, all samples were dialyzed against phosphate buffer (pH 7.4) and lyophilized.

Characterization of AGE-modified proteins

AGE-modified proteins were characterized by solid phase enzyme-linked immunosorbent assay (ELISA) and fluorospectrometry. An ELISA was performed as described by using the ELISA Starter Kit (Pierce, Rockford, IL, USA) [14]. In brief, AGE-modified proteins and control samples at various concentrations (0.001 to 20 µg/ml) were immobilized on 96-well polystyrene plates by incubation for 12 hours at 4°C. Each well was washed three times and nonspecific binding sites were blocked with blocking buffer composed of 1% bovine serum albumin (BSA). A polyclonal rabbit antiserum raised against KLH-AGE (gift of Dr. John Baynes, University of South Carolina) was reacted with the immobilized proteins at 1:2500 for one hour at
room temperature. A previous study had demonstrated that
anti-AGE antibody specifically recognized various AGE proteins,
but not early products such as Schiff bases and Amadori products
[15]. The wells were then washed with buffer, incubated with 100
μl of goat anti-rabbit IgG-peroxidase (dilution of 1:5000) (Pierce)
followed by addition of the substrate 2,2’-azino-di-3-ethylbenz-
thiazoline-6-sulfonic acid (ABTS). The absorbance at 405 nm was
measured on a micro-ELISA plate reader (TiterTek Multiskan,
Mec/340).

The fluorescence spectra of the AGE-modified proteins and the
control samples were measured at a protein concentration of 0.26
mg/ml in a fluorescence spectrophotometer (Aminco Bowman
Series 2) The maximum emission fluorescence intensities at 430
nm were determined upon excitation at 360 nm [16].

**Binding assays**

All buffers and substrate were obtained from an ELISA Starter
Kit as described above. Type I collagen incubated with or without
glucose and unincubated type I collagen were dissolved in 0.05 N
acetic acid, and each solution was adjusted to 10 μg/ml using the
coating buffer provided in the kit. Individual collagen solutions
(100 μl) were placed in 96-well polystyrene microplates and
incubated for 12 hours at 4°C in a moist chamber. The solution
was discharged, and the wells were rinsed three times with 100 μl
of wash buffer. Blocking buffer (100 μl) was added and allowed to
incubate for one hour. After emptying the plates, aliquots (100 μl)
of β₃m (25 to 100 μg/ml) incubated with or without glucose, and
unincubated β₃m, were added to the wells and incubated for one
hour at room temperature. After washing three times with wash
buffer, 100 μl of affinity purified sheep anti-human β₃m antibody
dilution of 1:2500; BiosPacific Inc., Emeryville, CA, USA) was
added, and the incubation was allowed to proceed at room
temperature for 60 minutes. Each well was rinsed three times, and
100 μl of rabbit anti-sheep IgG-peroxidase (dilution of 1:5000;
Pierce) was added. After incubation at room temperature for 60
minutes, the wells were washed, and 100 μl of ABTS was added
to each well. The reaction proceeded at room temperature for an
additional 30 minutes. The absorbance at 405 nm was measured
by using an ELISA reader as described above. Control studies
were performed using collagen-coated plates incubated with
blocking buffer alone or plates initially incubated with coating
buffer without collagen.

To test the interaction between the amount of AGE in collagen
and its binding capacity for β₃m, type I collagen-AGE and
unincubated type I collagen were mixed at various ratios. The
mixed collagen solution was then used at concentration of 10
μg/ml to coat the plate, and the plate was incubated with fresh
β₃m (75 μg/ml).

To determine the relative specificity of binding of β₃m to
collagen-AGE or unmodified collagen, two group of experiments
were performed. In the first group, plates were individually coated
with AGE-modified type I, II, III and IV collagens or their
unmodified forms, and the coated plates were incubated with 75
μg/ml of β₃m. In the second group, type I collagen-AGE or its
unmodified form were immobilized onto the plates and incubated
with β₃m, human serum albumin (HSA, Sigma) and human IgG
(H1gG, Pierce) at identical concentrations (75 μg/ml). The bind-
ing was determined by reaction with sheep anti-β₃m (dilution of
1:2500), sheep anti-HSA (dilution of 1:2500; Boehringer Mann-
heim, Indianapolis, IN, USA) and sheep anti-H1gG (dilution of
1:2500; Boehringer Mannheim), respectively.

**Nonenzymatic glycosylation of β₃m bound to collagen**

Under sterile conditions, all solutions were filtered through a
0.22 μm pore filter (Sartorius, Newton, NC, USA). Type I
collagen-AGE and unincubated type I collagen were coated onto
96-well microplates for 12 hours as described above. The wells
were rinsed three times. Sixteen microliters of β₃m solution (final
protein concentration 75 μg/ml) were then added and incubated
separately at 37°C for three weeks with 100 μl of D-glucose
solution (final concentration of glucose 0 to 100 mM in 100 mM
phosphate buffer) containing 200 U/ml penicillin, 70 μg/ml gent-
tamicin and 1.5 mM PMSF. The incubation solution was changed
every seven days by aspiration of the solution and replacement
with fresh incubation solution containing the original concentra-
tion of β₃m and glucose. At the end of the incubation period, the
wells were rinsed three times with wash buffer. One hundred
microliters of rabbit anti-KLH-AGE (1:2500) antibody was added
prior to incubation at room temperature for one hour. Each well
was rinsed three times, and 100 μl of goat-anti rabbit IgG-
peroxidase (dilution of 1:5000) was added. After incubation at
room temperature for one hour, the wells were washed, and
reacted with 100 μl of ABTS at room temperature for 30 minutes.
The absorbance at 405 nm was then measured by using an ELISA
reader as described above. Control studies were performed using
rabbit anti-human IgG (dilution of 1:2500; Boehringer Mann-
heim) and sheep anti-human β₃m (dilution of 1:2500; Boehringer Mannheim) instead of rabbit anti-KLH-AGE antibody.

**Statistical analyses**

All experiments were performed in triplicate. Continuous vari-
bles, expressed as mean ± SD, were compared using analysis of
variance (ANOVA). The Student-Newman-Keuls procedure was
used to evaluate pairwise comparisons. Two-tailed P values < 0.05
were considered statistically significant. Statistical analyses were
conducted with SAS (The SAS Institute, Cary, NC, USA).

**Results**

**Formation of AGE modified proteins**

After incubation for 30 days with 100 mM glucose, β₃m and the
four collagens individually reacted with anti-AGE antibody. Par-
allel incubations of β₃m or collagens without glucose, or the fresh
proteins, were without immunoreactivity. Comparison of AGE
formation between the four types of collagen showed that the
immunoreactivity of the type IV collagen product was significantly
weaker than that from the other three types of collagen (Fig. 1).

The reaction of the proteins with the anti-AGE antibody did not
reflect nonspecific binding, because the glycosylated proteins did
not react with anti-human Ig (data not shown). These data suggest
that β₃m-AGE and collagen-AGE were formed during the pro-
tein incubations with glucose.

The modification of β₃m and collagens to AGE was further
supported by their typical fluorescence spectra. β₃m and collagens
incubated with glucose showed intense fluorescence, with a major
excitation maximum at 360 nm for emission at 430 nm and a major
emission maximum at 430 nm upon excitation at 360 nm.
Fig. 1. The presence of AGE formation in collagens incubated with D-glucose. Microplates coated with 20 µg/ml of types I, II, III and IV collagen incubated with 100 mM D-glucose for 30 days were reacted with anti-AGE antibody. Data from three independent experiments are expressed as mean ± sd. ANOVA, P < 0.01. The immunoreactivity of type IV collagen differs significantly from types I, II and III.

contrast, control samples incubated without glucose and unincubated proteins did not fluoresce. In two experiments, after adjusting for the quantity of protein, AGE formation was 17% and 23% less from type IV than from type I.

Binding specificity of β2m to collagen-AGE

Microplates were coated with type I collagen-AGE and incubated with β2m or β2m-AGE. β2m readily bound to immobilized type I collagen-AGE. The quantity of β2m bound was significantly higher than that of AGE modified β2m (Fig. 2A; P < 0.0001). There was no significant difference in quantity bound of unincubated β2m compared with that incubated without glucose. At all concentrations of β2m, the quantity bound to type I collagen-AGE was significantly greater than to unmodified collagen (collagen-control) or fresh collagen (Fig. 2B; P < 0.0001).

Further support for the relative binding specificity of β2m for collagen-AGE was provided by mixing experiments in which plates were coated with incremental ratios of type I collagen-AGE and unincubated collagen (fixed final concentration of 10 µg/ml), to which a solution of 75 µg/ml of β2m was subsequently added. For ratios of 0, 0.25, 0.5, and 1.0 (collagen-AGE:unincubated collagen), absorbances of 0.57 ± 0.02, 0.83 ± 0.09, 0.99 ± 0.02, and 1.20 ± 0.02 were observed (P < 0.0001). Regression analysis showed a direct correlation between the ratio of collagen-AGE and the quantity of β2m bound (r = 0.97, P < 0.0001).

β2m was equally bound to all four types of collagen-AGE, as well as to the four types of unmodified collagen. Compared with β2m and HSA, IgG bound less well to collagen and collagen-AGE. The same pattern of increased binding to collagen-AGE relative to unmodified collagen was observed for all of these plasma proteins (Fig. 3).

Nonenzymatic glycosylation of collagen-bound β2m

Microplates coated with collagen-AGE or collagen were incubated for three weeks with incremental glucose concentration in the presence or absence of β2m (75 µg/ml). After incubation with β2m, both collagen-AGE and collagen coated plates reacted with the anti-β2m antibody, suggesting that β2m had bound to both immobilized collagen-AGE and unmodified collagen. No reaction was detected when the plates were incubated without β2m. Nonenzymatic protein glycosylation was detected by ELISA using the anti-AGE antibody. In the presence and absence of β2m, increased glucose concentrations were associated with increased
group. The structure, function, and distribution of the collagens in

immunoreactivity. Moreover, for any glucose concentration, the

immunoreactivity was greater for wells incubated with β2m. This

suggests that β2m bound to collagen-AGE underwent AGE formation in situ (Fig. 4A). In control experiments (Fig. 4B),

collagen-coated plates were incubated with incremental glucose

concentrations in the presence or absence of β2m (75 μg/ml).

Under these conditions, there was no significant difference in

AGE-protein formation with and without β2m.

Discussion

The affinity of β2m for other substances including native

collagens has been previously reported [17–19]. Spiegel and

coworkers have also observed that β2m is found associated with
collagen in the dermis of patients on dialysis [20]. Our results
demonstrated that β2m can bind to AGE-modified collagens in vitro, and the amount bound was significantly higher than to unmodified collagens. The quantity of β2m bound was dependent on the concentration of either β2m or AGE-modified collagen. Although we cannot exclude the possibility that the parallel accumulation of β2m and collagen-AGE in older dialysis patients may be fortuitous, the findings of a significant physical interaction between these proteins suggest an alternative pathobiologic explanation for the age-dependent occurrence of DRA. Retention of β2m due to chronic renal failure could be a prerequisite for the accumulation of β2m amyloid, while the increased amount of AGE in collagen seen with advanced age may be an important provocative factor in the occurrence of DRA.

We observed that β2m can interact with various types of
collagen-AGE. However, AGE formation in type IV collagen was

reduced compared with types I, II and III under identical incubation conditions. Unlike type I, II and III collagens (class I collagens), type IV collagen is a member of the class 3 collagen group. The structure, function, and distribution of the collagens in

this class differ substantially from those in class I. Class I collagen

forms the banded collagen fibers that are the major interstitial
collagen present in cartilage, skin, tendon, ligament, bone and

other connective tissues. They contain three alpha chains and are

first synthesized as procollagen. Class 3 collagen forms indepen
dent fiber systems that include basement membranes and beaded

filaments. Type IV collagen contains two alpha chains with a

complex globular domain at the carboxyl terminus. Carbohydrate

accounts for 10% of the mass of type IV collagen, a higher level

than is found in most other collagens [21]. The relationship

between the structures of the collagen classes and their relative

ability to form AGEs is unknown.

However, the relative ease of formation of class I collagen-

AGE may account for the observation that early in the course of

DRA, amyloid deposits are localized predominantly in joints and

periarticular bone. These structures are rich in class I collagen.

Later in the progression of DRA, amyloid deposition occurs in

basement membrane containing structures, such as the vascular

tissue and gastrointestinal tract, that contain type IV collagen as a

major component [7].

The close proximity of AGE-modified proteins with matrix

proteins such as collagen has been previously emphasized [22, 23].

Other plasma proteins such as HSA and IgG can also bind to
collagen-AGE in vitro, suggesting that the binding of β2m to
collagen-AGE in vivo may not be specific. Because immunohisto-

logical studies have demonstrated that β2m is a major constituent

of the amyloid fibrils in DRA [16], factors other than simple

binding and local accumulation of β2m to collagen-AGE must be
involved. It has been suggested that β2m amyloid is selectively

resistant to degradation, which may account for the relative

homogeneity of the deposits [24–26].

While the increase in circulating AGE-modified proteins ob-

served in diabetes is due to an increase in AGE production, it

appears to be due to decreased clearance in chronic renal failure
[27]. Makita et al showed that the serum AGE level of small-
molecular weight proteins was increased among non-diabetic

patients undergoing hemodialysis [27]. Furthermore, it has been
demonstrated that β2m in the amyloid deposits in DRA is
modified by AGE [16]. Thus, it has been hypothesized that the

increased circulating serum concentrations of β2m undergo gly-

cosylation into AGE with time, and β2m-AGE ultimately deposits

to form β2m amyloid in collagen-rich tissues [28]. We found that

native β2m, but not β2m-AGE, had a relatively higher affinity for

collagen-AGE or unmodified collagen. This suggests another

possibility. β2m may be initially bound to collagen-AGE or

collagen and modified locally to AGE as a subsequent event. The

demonstration that infusion of radiolabeled β2m rapidly localizes
to osteoarticular structures supports this hypothesis [29, 30]. Once

unmodified, short-lived plasma proteins become attached to

matrix proteins, their continuous accumulation in the tissue may

provide adequate time for these recruited proteins to form AGEs

[12].

To explore these hypotheses, we conducted experiments to
determine whether β2m could be modified to AGE when it was
bound to collagen-AGE. The nonenzymatic glycosylation of β2m
increased with higher glucose concentrations. Because the same
concentration of β2m had no effect on nonenzymatic glycosylation
in plates coated with unmodified collagen, this increased AGE-
formation could not be attributed to the promotive effect of β2m
on the AGE formation occurring in collagen. Therefore, we
High plasma and synovial fluid levels of \(\beta_2m\) due to decreased GFR

\[\beta_2m\text{ binds to collagen-AGE (class 1 > class 3)}\]

with preferential accumulation in osteo-articular structures

\[\beta_2m\text{ modified in situ to }\beta_2m\text{-AGE}\]

Enhanced accumulation of \(\beta_2m\) and other proteins through cross-linking

\[\beta_2m\text{ amyloid formation}\]

suggest that the increased AGE-formation after incubation of \(\beta_2m\) with collagen-AGE reflects the AGE modification of bound \(\beta_2m\).

No clinical correlation has been reported between DRA and diabetes mellitus [31]. Of particular clinical interest is the observation that \(\beta_2m\) bound to collagen-AGE was modified to AGE in the presence of physiologic concentrations of glucose. This may explain the clinical phenomenon of DRA in patients with non-diabetic chronic renal failure [2]. In addition, blood deoxyglucose levels are quite elevated in non-diabetic ESRD patient, and deoxyglucose may be a contributing carbohydrate facilitating \(\beta_2m\)-AGE formation in non-diabetic dialysis patients [32]. The absence of literature reporting diabetes mellitus as a risk factor for DRA is not surprising. ESRD patients with diabetes may not survive sufficiently long to develop DRA, that is, competing risks. The five-year death rate for diabetics with ESRD is twice that for non-diabetes (20% vs. 39% surviving, respectively) [33]. Furthermore, if deoxyglucose is a major provocateur for \(\beta_2m\)-AGE formation in vivo, the elevated blood levels in diabetics and non-diabetics would render both groups at equal risk for developing DRA.

Based on these results, the following mechanism is proposed (Fig. 5): \(\beta_2m\) initially and preferentially accumulates in osteo-articular tissue through binding to class 1 collagen-AGE, and then undergoes AGE modification. The new end product on this incorporated protein may then serve as an attachment site for additional \(\beta_2m\) or other plasma proteins and eventually form amyloid fibrils.

In conclusion, we demonstrate that \(\beta_2m\) binds to AGE modified collagens, and this incorporated \(\beta_2m\) can be modified further to AGEs. This may prove to be a potential recruitment mechanism in the pathogenesis of DRA.
Acknowledgments

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases grant #DK-49259-02 for Dr. William F. Owen, Jr. Dr. Fan Fan Hou was supported by a grant from National Medical Care, Inc., Waltham, Massachusetts, USA. We gratefully thank Lynda Herrera for administrative support.

Reprint requests to William F. Owen, Jr., M.D., Dialysis Unit Administrative Office, Brigham and Women’s Hospital, Dialysis, 75 Francis Street, Boston, Massachusetts 02115, USA.

References


