American Journal of Pathology, Vol. 162, No. 4, April 2003

Advanced Glycation End Products and Receptor for Advanced Glycation End Products in AA Amyloidosis

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Advanced glycation end products (AGEs) may be involved in either amyloidogenesis or complications related to amyloid. We hypothesized that AGEs may influence the pathogenesis of AA amyloidosis, and investigated the spatial and temporal relationship between AGEs, carboxy methyl lysine (CML), the AGE receptor (RAGE), and AA amyloid in humans and mice. Specimens from patients with AL and ATTR amyloidosis served as a control. Using immunohistochemistry, AGEs, CML, and RAGE were found within amyloid deposits, more commonly in AA amyloid than in AL amyloid and not in ATTR amyloid. Western blotting showed that multiple proteins (between 12 and >60 kd) are modified, but not the AA amyloid fibril protein itself. In the murine model of AA amyloidosis, we found a marked interindividual variability with respect to local and systemic CML levels, as well as to splenic RAGE transcription. Serum levels of CML correlated with the duration of the inflammatory response but not with amounts of splenic RAGE mRNA. Other as yet unidentified variables, especially of the heterogeneous group of AGEs, probably modulate transcription of RAGE and influence amyloidogenesis. CML serum levels, in turn, may prove useful in predicting patients at risk. *(Am J Pathol 2003, 162:1213–1220)*

Advanced glycation end products (AGEs) formed by nonenzymatic glycoxidation of proteins and lipids have been implicated in complications contributing to the increased morbidity and mortality of patients suffering from diabetes and uremia. Hyperglycemia in diabetic patients, and oxidative stress and carbonyl stress in uremic patients, contribute to the formation of AGEs, which are a chemically heterogeneous group of stable covalently bound and cross-linked adducts.[1–4] The detection of AGEs in prion plaques,[5] deposits of Aβ amyloid from Alzheimer patients,[6] hemodialysis-related Aβ2M amyloidosis,[7] and murine AApoAII amyloidosis[8] has indicated that nonenzymatic glycoxidation may also be involved in either amyloidogenesis or complications related to the deposition of amyloid. Amyloidoses are characterized by proteinaceous deposits of autologous origin that show specific structural and tinctorial properties. In AA amyloidosis, the acute-phase protein serum amyloid A (SAA) is the precursor of the AA fibril protein deposited in tissues. In the West, AA amyloidosis is most commonly related to rheumatoid arthritis.[9] Patients suffering from rheumatoid arthritis have significantly elevated serum and urine levels of AGEs, which correlate with parameters of disease activity such as C-reactive peptide, erythrocyte sedimentation rate, rheumatoid factor, and Lansbury index.[10–12] The activity of the inflammatory disease has also a major impact on amyloidogenesis,[13–15] and increased levels of AGEs and the possibility of developing AA amyloidosis are associated with the same risk factors. This raises the question whether AGEs may influence the pathology of AA amyloidosis.

The formation of AGEs is irreversible and the degree of modification correlates with the life span of the modified protein. AGEs are biologically active and may initiate a range of cellular responses including stimulation of monocyte chemotaxis, osteoclast-induced bone resorption, proliferation of vascular smooth muscle cells, aggregation of platelets, and stimulation of secretion of inflammatory cytokines, collagenase, and several growth factors.[4,16] The biological effect of AGEs is mediated, at least partly, by the receptor of advanced glycation end products (RAGE). RAGE is a multiligand, signal transduction receptor belonging to the immunoglobulin superfamily and it is expressed by a variety of cell types including endothelial cells, smooth muscle cells, lymphocytes, monocytes, and neurons.[16,17] Binding of ligands to

Supported by grants from the Deutsche Forschungsgemeinschaft, Bonn Bad-Godesberg, Germany (grant no. RO 1173/3); the German Government (grant no. BMBF-01ZZ9604); the Pinguin Stiftung, Düsseldorf, Germany; and the Wilhelm Vaillant-Stiftung, München, Germany.

Accepted for publication January 8, 2003.

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Table 1. Age and Sex Distribution, Underlying Disease, and Cause of Death of the Patients Studied

	AA amyloidosis	AL amyloidosis	ATTR amyloidosis
Number of cases	9	8	8
Total number of amyloidotic organs investigated	25	20	10
Age (years, range)	56 (35–70)	63 (50–73)	85 (78–99)
Sex (m:f)	6:3	3:5	5:3
Underlying disease (*n*)	Rheumatoid arthritis (5)	Plasmacytoma (3)	Senile cardiovascular amyloidosis (8)
	Rosai-Dorfman disease (1)	Immunocytoma (1)	
	Unknown (3)	Primary amyloidosis (4)	
Cause of death (*n*)	Cardiac failure (5)	Cardiac failure (4)	Intestinal infarction (1)
	Pulmonary thromboembolism (2)	Myocardial infarction (2)	Myocardial infarction (2)
	Renal failure (1)	Septic shock (1)	Pulmonary embolism (2)
	Acute appendicitis (1)	Gastrointestinal bleeding (1)	Panarteriitis nodosa (1)
			Cardiac failure (2)

RAGE[17,18] stimulates expression of RAGE itself,[17,18] and generates oxidative stress, synthesis and secretion of proinflammatory cytokines, and chemotaxis.[16–18] Thus, activation of RAGE propagates a chronic inflammatory disease state, that may further support the generation of AGEs. Yan and colleagues[19] have shown that canceling out the activation of cellular RAGE delayed the onset of reactive amyloidosis in mice, thus describing a putative pathophysiological pathway by which AGEs may influence amyloidogenesis.

To provide further evidence for the hypothesis that AGEs and RAGE may influence the pathogenesis of AA amyloidosis, we investigated the spatial and temporal relationship between AGEs, carboxy methyl lysine (CML), RAGE, and AA amyloid in humans and mice. Specimens from patients with light chain-associated (AL) amyloidosis and senile cardiovascular (ATTR) amyloidosis served as a control.

Materials and Methods

Patient Selection

Fifty-five archived, formalin-fixed, paraffin-embedded, autopsy specimens from a series of 25 patients were used in this study. These patients have been described previously when histological examination demonstrated the presence of generalized amyloidosis, either AA, AL, or ATTR amyloidosis.[20,21] Age, sex distribution, and underlying diseases are summarized in Table 1. The classification of amyloid was based on immunohistochemistry and clinical history as described elsewhere.[21–23] Nine patients had suffered from AA amyloidosis, eight from AL amyloidosis, and eight from senile cardiovascular ATTR amyloidosis.[20,21] The following organ specimens were available for use in this study: liver (from 20 patients), kidney (17 patients), spleen (14 patients), and heart (4 patients). Variable amounts of amyloid were present in all of the specimens as vascular and interstitial deposits.

In addition, unfixed splenic tissue containing amyloid was available from three of the above-mentioned patients with generalized AA amyloidosis[14,24] and this was used for the preparation of amyloid fibril proteins as described previously[25] using ~6 g of amyloidotic tissue.[24]

Induction of AA Amyloidosis in Mice

Six- to 8-week-old BALB/c female mice were purchased from Harlan Winkelmann GmbH (Borchen, Germany). The mice were fed a normal chow diet and water *ad libitum*. AA amyloidosis was induced by daily subcutaneous injections of 7% azocasein for 20 days. The animals were sacrificed in an atmosphere of CO_2 after 5 (five animals), 10 (five animals), 15 (five animals), and 20 (five animals) injections. Five mice received no injections and served as negative controls. The mice were bled by cardiac puncture and serum was obtained by centrifugation for 10 minutes at 2500 $\times$ *g* (4°C) before storage at −80°C until required. The spleens were removed and half of each organ was frozen immediately in liquid nitrogen and stored at −80°C until further use. The other half of the organ was fixed in 5% paraformaldehyde, pH 7.4, in phosphate-buffered saline (PBS), for 24 to 48 hours and then embedded in paraffin.

Preparation of AGE-Modified Proteins

AGE-modified keyhole limpet hemocyanin (AGE-KLH) was produced by incubating KLH (20 mg/ml; Sigma, Deisenhofen, Germany) with 1 mol/L of glucose in 200 mmol/L of PBS, pH 7.4, containing 0.025% NaN_3 at 37°C for 3 months. Bovine serum albumin, human fibronectin (0.5 μg/ml), and human laminin (1 mg/ml; all from Sigma) were glycosylated nonenzymatically in a similar way for a period of 1 month. Collagen IV (Sigma) was dissolved in 10 mmol/L of sodium acetate, pH 5.5, and diluted with 10 mmol/L of Tris-HCl, pH 7.5, containing 100 mmol/L of NaCl, and then at a concentration of 1 mg/ml incubated with glucose as described above for 1 month. Incubation of KLH at 37°C in the absence of glucose served as a control. Surplus glucose was removed by dialysis in PBS (pH 7.4) using a cellulose dialysis membrane Spectra Por 4, MW 12,000 to 14,000 (Spectrum, Gardena, CA). Successful glycosylation was tested using fluorospectrometry. Fluorescence was measured at excitation and emission wavelengths of 398 and 450 nm, respectively, with a LS-5 Perkin-Elmer spectrofluorimeter (Perkin-Elmer, Munich, Germany).

Generation and Characterization of Polyclonal Anti-AGE Antibodies

Rabbits were immunized with 20 mg/ml of AGE-KLH according to a standardized immunization protocol (Eurogentec, Herstal, Belgium). IgG was obtained by using HiTrap protein G columns (Amersham Pharmacia Biotech, Freiburg, Germany). The specificity was tested using an enzyme-linked immunosorbent assay (ELISA) using either AGE-modified bovine serum albumin, collagen type IV, fibronectin, KLH, or laminin. Anti-AGE recognized KLH, AGE-bovine serum albumin, AGE-collagen, AGE-KLH, and AGE-laminin, but not native bovine serum albumin, collagen, or laminin (data not shown).

Histochemistry and Immunohistochemistry

Paraffin sections were stained with hematoxylin and eosin. The presence of amyloid was demonstrated by the appearance of green birefringence from alkaline alcoholic Congo red staining under polarized light.[26] Immunostaining was performed with antibodies directed against AGE (polyclonal, anti-AGE, dilution 1:20), CML (monoclonal, clone 4G9, dilution 1:1000; Roche Diagnostics GmbH, Penzberg, Germany),[27] human RAGE (monoclonal, dilution 1:20),[28] and human AA amyloid (monoclonal, clone mc_1, dilution 1:500; DAKO, Hamburg, Germany). Before immunostaining with anti-AGE the specimens were pretreated with 10 mmol/L of ethylenediaminetetraacetic acid (2 $\times$ 10 minutes, 450 W microwave oven). Before immunostaining with anti-CML the specimens were pretreated with Proteinase K (30 minutes at 37°C). Immunostaining was performed as described elsewhere.[24] Specimens of formalin-fixed and paraffin-embedded arteriosclerotic vessels served as positive controls for AGE, CML, and RAGE. The specificity of immunostaining was controlled using specimens containing known classes of amyloid (anti-AA amyloid). Incubation with preimmune serum and omission of primary antibodies served as negative controls.

Gel Electrophoresis and Western Blotting

Quantities of between 50 and 200 mg of tissue, which had been stored at −80°C, were homogenized in 0.5 to 2.0 ml of lysis buffer (4 mol/L urea, 0.5% sodium dodecyl sulfate, 62.5 mmol/L Tris, pH 6.8, 1 mg/ml protease inhibitor cocktail; Sigma) using an Ultra-Turrax T8 (IKA-Labortechnik, Staufen, Germany). The homogenate was centrifuged at 11,000 $\times$ *g* for 10 minutes at 4°C. Aliquots of 25 μg or 50 μg were resolved in 10 to 16.5% two-phase polyacrylamide gels and visualized by staining with Coomassie blue. Proteins on unstained polyacrylamide gels were transferred onto a polyvinylidene difluoride membrane (Immobilon-P^{SQ}; Millipore, Bedford, MA) and blocked overnight with Roti-Block (Carl ROTH GmbH, Karlsruhe, Germany). Immunostaining was performed using anti-AA amyloid (monoclonal, clone mc_1, dilution 1:500; DAKO), anti-AGE (dilution 1:500), anti-CML (1:250), or biotinylated anti-human serum albumin (monoclonal, 1:1000; Roche Diagnostics GmbH). Alkaline phosphatase-conjugated second or anti-rabbit/anti-mouse antibodies (dilution 1:1000, room temperature, 60 minutes; DAKO) or alkaline phosphatase-conjugated streptavidin (1:3000, room temperature, 60 minutes; Rockland, PA) served as bridging reagents and 1-Step Tetranitroblue tetrazolium/5-bromo-4-chloro-3-indolyphosphate (Pierce, Bonn, Germany) as chromogen.

Competitive ELISA for the Quantification of CML

The concentration of CML in murine serum and spleen homogenates was quantified using a competitive ELISA developed by Roche Diagnostics GmbH.[29] Samples from serum or tissue homogenate were diluted (serum, 1:20; tissue homogenate, 1:200) in washing buffer and pretreated with Proteinase K (1 mg/ml, 37°C for 2 hours; Roche Diagnostics GmbH).

Polymerase Chain Reaction of Murine RAGE mRNA

Total RNA was isolated from small pieces (<10 mg) of frozen spleen. After homogenization using an Ultra-Turrax T8 (IKA-Labortechnik), the homogenate was prepared with the RNeasy mini kit according to manufacturer's instructions (Qiagen, Hilden, Germany). The reverse transcription into cDNA was performed using the Omniscript RT Kit (Qiagen), oligo$(dT)_{12\text{-}18}$ primers (Promega, Mannheim, Germany), RNaseOUT recombinant ribonuclease inhibitor (LifeTechnologies, Karlsruhe, Germany), and 1 μg of RNA per sample as a template. Two μl of the cDNA template were used for the polymerase chain reaction (PCR) analysis. The master mix used contained the following per reaction (50 μl): 1.5 mmol/L $MgCl_2$, 200 μmol/L of each dNTP, 50 pmol of each primer, and 2.5 U *Taq*DNA polymerase (*Taq*PCR core kit from Qiagen). The sequences of the primers for the murine RAGE-mRNA were: 5′-CAGGGTCACAGAAACCGG-3′ (upstream), and 5′-ATTCAGCTCTGCACGTTCCT-3′ (downstream). The standard temperature profile included an initial denaturation for 2 minutes at 92°C, followed by 35 cycles of denaturation at 92°C for 30 seconds, annealing at 60°C for 90 seconds, and an extension at 72°C for 60 seconds. The products were transferred onto a 1.5% agarose gel labeled with ethidium bromide, and analyzed by transillumination. The PCR product was isolated from the gel using the MinElute extraction kit from Qiagen and sequenced on the capillary sequencer 310 from ABI Prism (Foster City, CA).

Quantification of Murine RAGE mRNA by Real-Time Reverse Transcriptase-PCR

Assays were performed on the LightCycler (Roche Diagnostics GmbH). Several dilutions of plasmids containing the cDNA fragments of RAGE were used as internal controls. For plasmid construction, the cDNA fragments were amplified using the primer described above and

inserted into the pCR2.1-TOPO vector (Invitrogen, Groningen, The Netherlands). The copy number of the resulting plasmids was calculated after DNA quantification. All reactions contained 2 mmol/L of $MgCl_2$, 10 pmol of each primer, 2 μl 10× LightCycler-DNA Master SYBRGreen I (Roche Diagnostics GmbH), and 13.2 μl of distilled water to adjust the final volume (20 μl). Two μl of the cDNA template were added to the prepared capillaries. The standard temperature profile started with an initial denaturation at 95°C for 30 seconds, followed by 40 cycles of denaturation at 95°C for 1 second, annealing at 62°C for 5 seconds, and an extension at 72°C for 15 seconds.

The quantification of RAGE mRNA was performed as follows: β-actin (upstream primer: 5′-TGACGGGGTCACCCACACTGTGCCCATCTA-3′, downstream primer: 5′-CTAGAAGCATTTGCGGTGGACGATGGAGGG-3′) was used as a housekeeping gene, and the RAGE mRNA/β-actin mRNA ratio was calculated for all samples. Each sample was measured in triplicate.

Statistical Analysis

All values are expressed as mean ± SD. The chi-square test and the unpaired Student's *t*-test were used where appropriate. The Pearson correlation coefficient was used to determine the relationship between metric parameters. A value of $P < 0.05$ was considered to be statistically significant.

Results

Prevalence of AGEs and RAGE in Human AA Amyloidosis

AGEs were found immunohistochemically in every patient (100%) with AA amyloidosis within amyloid deposits (Figure 1). It was present in deposits of the kidney, liver, and spleen. AGEs were often present only in some deposits, whereas others within the same specimen were negative (Figure 1). Only a single liver specimen showed amyloid deposits completely devoid of AGEs (Table 2). AGEs were also present in tubulus epithelium of kidneys, on erythrocytes, in the interstitium, and within vessel walls. In seven (78%) patients AGEs were identified as CML by using a specific antibody (Figure 1). CML was found most commonly within the amyloid deposits of the kidney (100%), followed by spleen (67%) and liver (11%; Table 2). It was interesting to note that CML was also strongly expressed by cells lining AA amyloid deposits (Figure 1, inset).

RAGE displayed a spatial relationship with amyloid in all of the cases studied. RAGE was found either within the amyloid deposits (Figure 1) or adjacent to the deposits where it was extracellular or intracellular. Intracellular immunostaining was confined mainly to myocytes of vessel walls, endothelial cells, and macrophages, which were either attached to or enclosed by amyloid deposits. Within amyloid deposits, RAGE again was found most commonly in the kidney (86%), followed by spleen (77%) and liver (77%; Table 2). Immunostaining for RAGE was more extended and more even as compared to AGE and CML (Figure 1).

Detection of AGEs in Homogenates of Human AA Amyloidosis

Western blots were performed for further analysis of the nature of the AGE-modified compounds associated with AA amyloid. Unfixed samples of amyloidotic spleens obtained from three of the nine patients with AA amyloidosis studied immunohistochemically were homogenized and submitted to electrophoresis and Western blotting. Using an antibody directed against AA amyloid, immunoreactive bands were detected in the crude tissue homogenates of all three patients (Figure 2). All three patients showed bands at ~6 kd, which are consistent with the molecular weight of the AA amyloid fibril proteins (Figure 2), whereas patients 2 and 3 showed additional bands at ~12 kd representing the precursor protein serum amyloid A. Immunoblotting of the homogenates with anti-AGE showed multiple immunoreactive bands in all cases (Figure 2) ranging from 17 kd to >60 kd were immunoreactive for AGE (Figure 2). None of these bands had the same size as the fibril proteins. Immunoblotting of the homogenates with anti-CML showed similar bands in all cases ranging from 12 kd to >60 kd (Figure 2), some of these bands were attributable to nonenzymatically glycosylated human serum albumin (data not shown).

Prevalences of AGEs and RAGE in Human AL and ATTR Amyloidosis

AGEs were found significantly less commonly in AL amyloidosis ($P < 0.01$). They were present within the deposits of five patients (63%), including liver (63%), kidney (57%), and spleen (40%; Table 2). The staining pattern of anti-AGE within AL amyloid was similar to AA amyloid. In five patients AGEs were specified as CML (Table 2) and was found most commonly in the spleen (80%) followed by kidney (43%; Table 2). CML was not found in the liver. In contrast to AA amyloid, CML was not observed in cells lining AL amyloid deposits (Figure 1). The different prevalences of CML between AA and AL amyloid were statistically not significant.

RAGE was also present in AL amyloid deposits, but significantly less common than in AA amyloid ($P < 0.01$; Table 2). Neither AGE, CML, nor RAGE were found in deposits of ATTR amyloid (Table 2).

CML and RAGE in the Murine Model of Reactive Amyloidosis

The immunohistochemical and biochemical studies provide evidence for high prevalences of AGEs, CML, and RAGE in human AA amyloid deposits. However, they provide no information about the order of events and a putative temporal correlation. To investigate the occurrences and expression profile of CML and RAGE, respectively, during amyloidogenesis, AA amyloidosis was in-

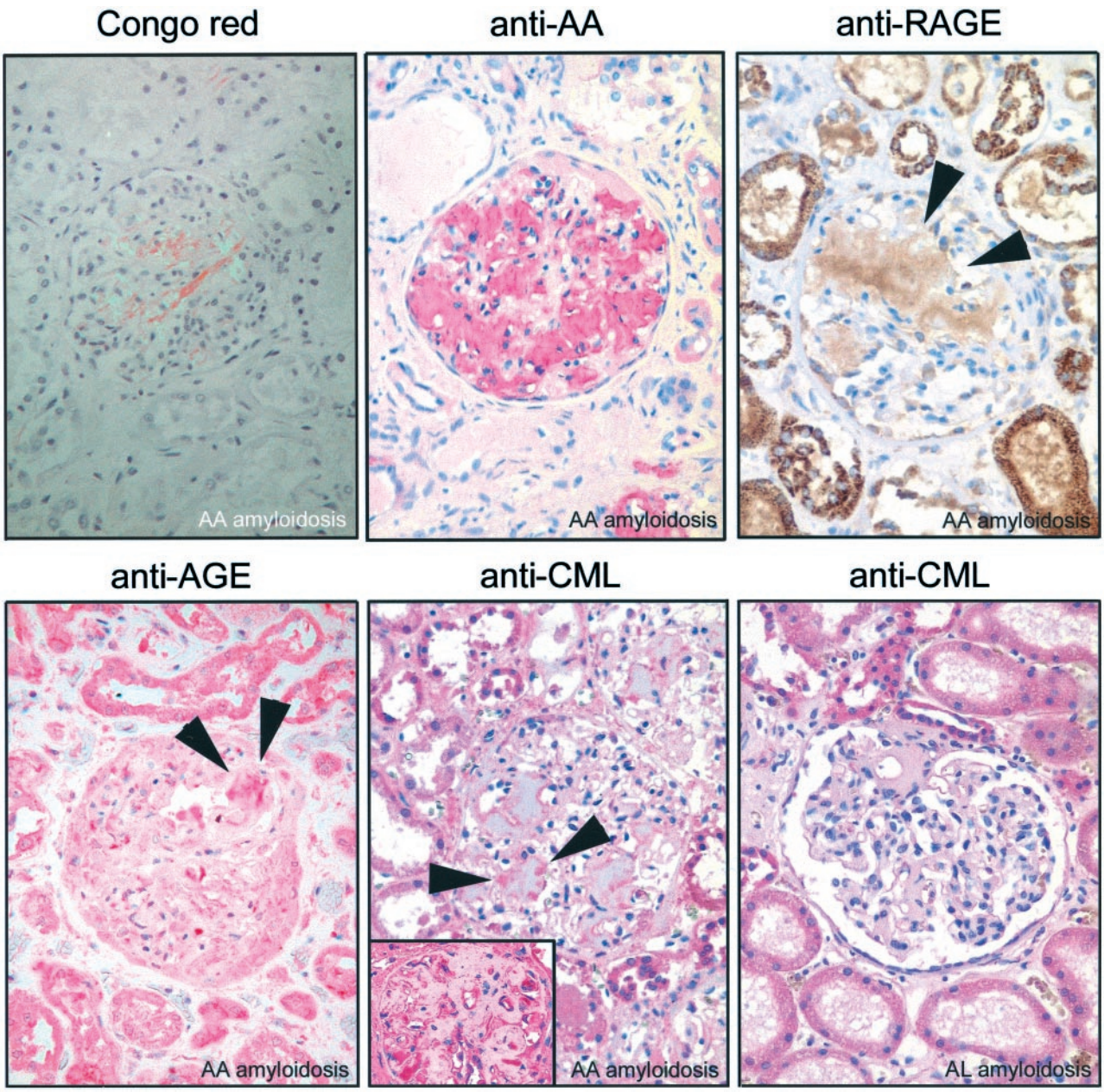

Figure 1. Kidney specimens from a patient with generalized AA amyloidosis show AGE, CML, and RAGE within amyloid deposits (**arrowheads**). CML was also found in cells lining amyloid deposits (**inset**). CML was found less commonly in AL amyloid. Congo red staining in polarized light. Immunostaining with anti-AA amyloid, anti-AGE, anti-CML, and anti-RAGE; hematoxylin counterstain. Original magnifications: ×20; ×40 (**inset**).

duced in amyloid-susceptible BALB/c-mice. A standard induction protocol with daily subcutaneous injections of azocasein resulted in the formation of amyloid. Amyloid occurred in the spleen as early as after 15 injections (two of five mice) and was present in all mice after 20 injections. A competitive ELISA was performed to measure the relative amounts of CML in serum and spleen during amyloidogenesis. The amount of CML in the serum showed a V-shaped pattern with the lowest level after 15 injections and the highest after 20 injections (Table 3). The difference in the serum levels of CML between 15 and 20 injections proved to be significant ($P < 0.05$). In contrast to the serum, the concentration of CML in the spleen showed only mild variations throughout time (Table 3) and it did not correlate with the number of injections given ($P = 0.88$) or with the serum level of CML ($P = 0.68$).

The amount of RAGE mRNA was quantified in murine spleens using real-time PCR and showed a V-shaped pattern: the amount of mRNA decreased after five injections and increased significantly thereafter (5 *versus* 10 injections, $P < 0.01$) with a peak after 15 injections (5 *versus* 15 injections, $P < 0.05$) (Table 3). No further significant correlations were found between the relative amount of RAGE mRNA and the number of injections given. The relative amount of RAGE mRNA did not cor-

Table 2. Distribution Pattern of AGEs, CML, and the RAGE within AA, AL, and ATTR Amyloid Deposits

	AGE *n* (%)	CML *n* (%)	RAGE *n* (%)
AA amyloidosis			
Kidney ($n = 7$)	7 (100)	7 (100)	6 (86)
Liver ($n = 9$)	8 (89)	1 (11)	7 (77)
Spleen ($n = 9$)	9 (100)	6 (67)	7 (77)
Total ($n = 25$)	24 (96)	14 (56)	20 (80)
AL amyloidosis			
Kidney ($n = 7$)	4 (57)	3 (43)	2 (29)
Liver ($n = 8$)	5 (63)	0	2 (25)
Spleen ($n = 5$)	2 (40)	4 (80)	0
Total ($n = 20$)	11 (55)	7 (20)	4 (20)
ATTR amyloidosis			
Kidney ($n = 3$)	0	0	0
Liver ($n = 3$)	0	0	0
Heart ($n = 4$)	0	0	0
Total ($n = 10$)	0	0	0

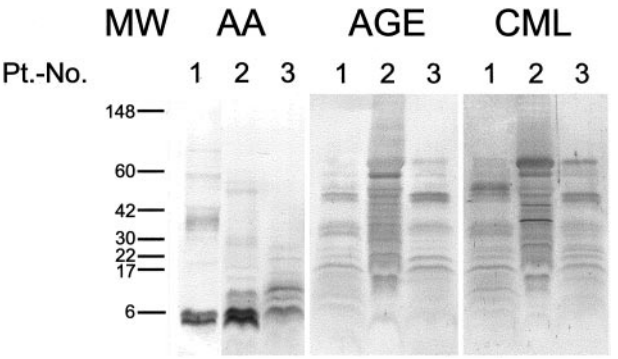

Figure 2. Unfixed, crude amyloidotic spleen homogenates from three patients with generalized AA amyloidosis were homogenized and separated by gel electrophoresis. Western blotting with an antibody directed against human AA amyloid identified bands of ~6 and 12 kd. Immunolabeling with anti-AGE and anti-CML showed multiple bands ranging from 12 to >60 kd. Molecular weight standards in kd (MW). CB, Coomassie blue staining; Pt.-No., patient number; AA, Western blotting with anti-AA amyloid; AGE, anti-AGE; and CML, anti-CML.

relate with the levels of CML in the serum ($P = 0.901$) or spleen ($P = 0.226$).

It is important to note that CML levels in serum and spleen as well as the amount of RAGE mRNA showed great, nonsystematic interindividual variability, as documented by the high SD, irrespective of the number of injections applied.

Discussion

Prevalence and Pathogenesis of AGEs in Amyloidosis

In the present study we investigated the spatial and temporal relationships between the presence of AGEs and AA amyloid in human and murine tissues and compared it with AL and ATTR amyloidosis. Using immunohistochemistry, we found AGEs that were spatially related to human amyloid deposits and occurred significantly more commonly in AA than AL or ATTR amyloidosis. Contradicting results have been obtained regarding the occurrence of AGEs in human AA amyloidosis, which is related most likely to the heterogeneity of AGEs. Using monoclonal antibodies Niwa and colleagues[30] were unable to detect imidazolone in AA amyloid deposits. Uesugi and colleagues[31] applied antibodies directed against CML and pyrraline and found only CML in renal amyloid deposits. We applied polyclonal antibodies, which are more likely to recognize a broad spectrum of different AGEs in AA amyloidosis.[32] We also found CML in AA and AL amyloidosis, confirming the observation made by Uesugi and colleagues.[31] However, CML was less common than AGEs in general, as demonstrated here by comparing the staining of polyclonal and monoclonal antibodies. Altogether these observations show that AGEs in AA and AL amyloid constitute a heterogeneous group, and that CML contributes only partly to AGEs formed in AA and AL amyloid.

In our series of specimens, the increased prevalences of AGEs and CML in AA amyloid are attributable to the underlying disease and not to patient's age: most patients with AA amyloidosis had suffered from a chronic or recurrent inflammatory disease known to be associated with an increased serum concentration of AGEs, including CML, and, on average, patients with AA amyloidosis were younger than those with AL and ATTR amyloidosis. The presence of AGEs in some patients with AL amyloidosis may also be related to the underlying disease; the majority had suffered from either multiple myeloma or primary amyloidosis disease conditions that are known to cause chronic renal failure and chronic recurrent infections that may result in the generation of AGEs. We did not find AGEs in ATTR amyloid, again indicating that patient's age cannot account for the occurrence of AGEs in AA and AL amyloidosis. Patients with ATTR amyloid had the highest mean age in our series.

Table 3. AA Amyloidosis Was Induced in BALB/c Mice Using Daily Injections of 7% Azocasein

Number of injections	CML serum (ng/mg protein)	CML spleen (ng/mg protein)	Copies RAGE mRNA/copies β-actin mRNA $\times 10^5$
0 ($n = 5$)	8.25 ± 2.87	10.03 ± 3.14	31.14 ± 65.33
5 ($n = 5$)	6.65 ± 1.82	9.17 ± 2.70	1.91 ± 1.10†
10 ($n = 5$)	7.83 ± 2.29	8.13 ± 3.31	10.25 ± 5.80†
15 ($n = 5$)	4.86 ± 2.21*	9.35 ± 2.38	60.06 ± 46.98†
20 ($n = 5$)	8.84 ± 2.10*	9.14 ± 1.27	52.86 ± 57.88

*Fifteen *versus* 20 injections; $P < 0.05$.
†Five *versus* 10 injections; $P < 0.01$; 5 *versus* 20 injections; $P < 0.05$.
Levels of CML in serum and spleen homogenates as well as the relative amount of mRNA of the RAGE were measured as indicated.

Pathophysiological Role of AGEs and RAGE in AA Amyloidosis

AGEs may influence the pathogenesis of an amyloid disease in different ways. Modification of the precursor or fibril protein may result in altered biological and biophysical properties for the propagation of fibril formation. Using tissue homogenates and Western blotting, we were able to show that neither the precursor nor the fibril protein of human AA amyloidosis were modified by AGEs or CML. Thus, if AGEs or CML do have an impact on the pathogenesis of AA amyloidosis, this effect is not mediated by the modification of SAA or AA fibril proteins.

AGEs may influence amyloidogenesis by activating RAGE. Binding of ligands to RAGE leads to translocation of nuclear factor-kappa B via transient activation of tyrosine phosphorylation, extracellular signal-regulated kinases 1 and 2, and p38 mitogen-activated protein kinase.[19,33,34] Yan and colleagues[19] have shown that RAGE is able to influence the pathogenesis of AA amyloidosis; canceling out the activation of cellular RAGE delayed the onset of reactive amyloidosis in mice. They also demonstrated that AA fibril proteins and the amyloidogenic variant of murine SAA (SAA1.1) are ligands for RAGE.[19] We found RAGE to be spatially related to amyloid deposits in all patients with AA amyloidosis, which confirms a previous observation made in a single case.[19] Again, it was found significantly more commonly in AA than in AL or ATTR amyloid. RAGE was not only cell bound, but also present extracellularly within the deposits. Because RAGE was shown to bind AA fibril proteins it may become enriched within AA amyloid deposits.[19] Similar to AGEs, the presence of RAGE may also be related to the underlying disease.

CML binds to cellular RAGE and may influence the expression profile of RAGE[33] and, indeed, we found CML highly expressed in cells lining amyloid deposits. By using a murine model, we investigated the temporal relationship between CML levels in serum and spleen, and the expression profile of RAGE mRNA during amyloidogenesis. Interestingly, CML was present in healthy animals and CML-levels in the serum decreased after an inflammatory stimulus was set. Thus, during the early course of a chronic inflammatory stimulus, adaptive mechanisms seem to reduce CML load and RAGE expression. The decreased amount of CML, in turn, may be related to increased clearance mediated by RAGE. It is interesting to note that the low serum levels of CML after 15 injections were associated with the highest amounts of RAGE mRNA in the spleen. However, with an ongoing inflammatory stimulus, which then leads to *de novo* formation of AGEs, the serum CML level increases significantly while amyloid is deposited in the spleen. Thus serum levels of CML may indicate duration of the disease. Future studies are required to show whether serum levels of AGE or CML can be used to predict severity or duration of a chronic inflammatory disease and thus predict the occurrence or progression of AA amyloidosis, as it was demonstrated for Aβ2M amyloidosis.[35]

Statistical analyses failed to show any correlation between serum or splenic CML levels and amounts of RAGE mRNA, indicating that transcription of RAGE does not depend on CML levels only. At least three different ligands, which occur during amyloidogenesis, compete for binding to RAGE: SAA1.1, AA fibril protein, and CML.[19] Indeed, AA fibril proteins and CML have similar kd values (between 60 and 76 nmol/L)[16,19,33] for binding to RAGE. Apart from CML, other AGEs are likely to bind to RAGE and may modulate its expression,[33] which, altogether, may explain why the transcription levels of RAGE demonstrate such a variability. Furthermore, different receptors are capable of binding and/or clearing AGEs, including the macrophage scavenger receptor classes A and B, 80 K-H phosphoprotein, oligosaccharyl transferase-48, and galectin-3,[36,37] which all may influence local and systemic concentrations of AGEs. However, it is important to note that the highest amount of RAGE mRNA was found when amyloid started depositing in the spleen.

In our series, immunostaining of amyloid with anti-AGE was sparse, heterogeneous, and present in only some deposits, whereas others, within the same specimen, were immunonegative. Thus, AGEs constitute just a minor component of AA amyloid deposits and it is even not the fibril protein being modified. These observations suggest that the presence of AGEs, per se, is no prerequisite for the formation of AA amyloid fibrils themselves. Similarly, Niwa[38] proposed that modification of β2-microglobluin in dialysis related Aβ2M amyloidosis occurs after amyloid has been formed.

In summary, we show that AGEs and RAGE are spatially related to AA amyloid, and that high-molecular weight compounds are modified in AA amyloid, but not the AA fibril protein itself. We found a marked interindividual variability with respect to local and systemic CML levels as well as to local RAGE transcription, making it difficult to find simple explanations for a putative pathophysiological link between CML, RAGE, and AA amyloidosis. Other as yet unidentified variables, particularly belonging to the heterogeneous group of AGEs, may modulate transcription of RAGE and influence amyloidogenesis. Nevertheless, CML serum levels may prove useful in predicting patients at risk.

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