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NEUTROPHIL PROTEASES ASSOCIATED WITH AMYLOID FIBRILS

Phillip J. Stone*[a], Josep M. Campistol**, Carmela R. Abraham* †, Orville Rodgers†, Tsuranobu Shirahama†, and Martha Skinner†

*Department of Biochemistry, Boston University School of Medicine, Boston,MA 02118

** Hospital Clinic, University of Barcelona, Spain

†Department of Medicine, Arthritis Center, Boston University School of Medicine, Boston,MA 02118

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Summary: We found significant amounts of enzymatic activity characteristic of the human neutrophil proteases, elastase and cathepsin G, associated with isolated amyloid fibrils from patients with five different types of systemic amyloidosis. Amyloid deposits in tissue sections from the patients with amyloid A, amyloid transthyretin and amyloid β_2-microglobulin amyloidosis also stained positive with antiserum to elastase and cathepsin G. Elastase and cathepsin G, found in the azurophilic granules of the neutrophil and, to a lesser extent, the monocyte, may become associated with amyloid precursor proteins before or during the formation of amyloid fibrils. This may occur in an extracellular inflammatory microenvironment or in a phagolysosome and play a role in the formation of the fibrils.

Proteases are thought to play an important role in the formation of amyloid fibrils from precursor proteins. Studies have shown the close proximity of fibrils to lysosomes in an experimental model [1]. More recently the mutant transthyretin (TTR) has been shown to be less stable in a low pH environment such as that found in a lysosome [2]. The identification of the individual protease(s) or the site of proteolytic processing remains uncertain. Human neutrophil elastase (HNE)(EC 3.4.21.37) has been identified as a neutrophil protease that is normally capable of degrading serum amyloid A [3,4]. We have reported that HNE appears to be intimately associated with fibrils of primary (AL), secondary (AA) and hereditary (ATTR) amyloid as they

[a] To whom correspondence should be addressed (FAX: 617-638-5339).

ABBREVIATIONS USED: AA, amyloid A or secondary amyloid; AApo A-1, amyloid Apo A-1; Aβ_2M, amyloid β_2-microglobulin; AL, amyloid light chains or primary amyloid; ATTR, amyloid transthyretin; β_2M, β_2-microglobulin; Cat G, human neutrophil cathepsin G; HNE, human neutrophil elastase; PMSF, phenyl methyl sulfonyl fluoride; TTR, transthyretin.

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are isolated from the tissue [5]. We have now found evidence for the presence of a second neutrophil protease, cathepsin G (Cat G)(EC 3.4.21.20), associated with amyloid fibrils, further suggesting a role for neutrophils or monocytes in amyloidogenesis. Cat G, along with HNE, is found in the azurophilic granules of the neutrophil and, to a lesser extent, monocytes. We also assessed synovial fluid and β_2-microglobulin (β_2M) deposits from synovial spaces for the presence of activated collagenase.

MATERIALS AND METHODS

Preparation of amyloid fibrils and synovial fluid

Amyloid fibrils were isolated from amyloid-rich human tissues according to published methods [6]. Fibrils were also isolated from amyloidotic spleens of CBA/J mice in whom amyloidosis was induced with casein as previously described [1]. Synovial fluid was obtained from the knees of hemodialysis patients with severe forms of Aβ_2M amyloidosis (carpal tunnel syndrome and amyloid arthropathy). Amyloid deposits were demonstrated by Congo red staining in the sediment of the synovial fluid after centrifugation.

Measurement of cathepsin G and elastase activity

HNE and Cat G were purified as previously described [7]. Cat G activity was measured spectrophotometrically at 410 nm using the chromogenic substrate, Succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine paranitroanilide (60 mM) in 1-methyl-2-pyrrolidinone [8]. Fibrils (4 mg) were incubated in a cuvette containing 2.85 ml of 0.1 M Tris, pH 8.3, 0.01 % sodium azide and 0.15 ml substrate solution. Periodically, the cuvettes were covered and inverted twice. Depending on the amount of activity present, the cuvettes were incubated up to 3 hr at 25°C. Incubations were also carried out with fibrils in the presence of a serine protease inhibitor, phenylmethyl sulfonyl fluoride (PMSF) (dissolved in ethanol, 100 mg/ml, 20 µl added per sample).The effect of HNE on the Cat G substrate was assessed as follows. A rate of hydrolysis of the Cat G substrate in the presence of fibrils was determined. The HNE inhibitor, Succinyl-L-alanyl-L-alanyl-L-prolyl-L-valine chloromethyl ketone (0.3 mM) (Enzyme Systems Products, Livermore, CA) was then added and the rate of hydrolysis measured again.

Elastase activity was measured by incubating 4 mg of fibrils with [^{3}H]-insoluble neck ligament elastin as substrate for 4 hr at 37° in phosphate-buffered saline, pH 7.35 [9].

Collagenase assay

Collagenase activity was measured using [^{3}H]-fibrillar collagen prepared from neonatal rat smooth muscle cell cultures [10]. Briefly, the cultures were incubated with L-[4,5-^{3}H] lysine for 1 day, the media replaced with fresh media containing non-radioactive lysine and then cultured for another 6 wk, before harvest. Collagen was prepared by digestion of the cell layer with porcine pancreatic elastase and washed. The residue exhibits the amino acid composition of collagen. The purified [^{3}H]-collagen substrate (1000 cpm/µg) was suspended in 0.05 M Tris, pH 7.5, 0.005 M $CaCl_2$, aliquoted into tubes, washed once, and fibrils, synovial fluid or purified enzymes were added. Incubation was for 18 h at 32° C. The tubes were agitated, centrifuged and 10% of the supernatant was assessed for [^{3}H] by liquid scintillation spectrometry. Bacterial collagenase used as a positive control for collagenolytic activity was purified as described by Hu and coworkers [11].

Immunohistochemistry

Four micron serial sections were cut from formalin-fixed paraffin-embedded tissues. One section was stained with Congo red and hematoxylin and examined under polarized and non-polarized light for Congo red staining material with green birefringence characteristic of amyloid. Adjacent sections were used for immunohistochemical preparations. Sections were incubated in 10 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 0.05% Tween 20 with polyclonal rabbit antisera to HNE (1:250) or Cat G (1:250) (Calbiochem) and visualized using an avidin-biotin complex method (Vector Laboratories) [12]. Controls included serial sections that were incubated with non-immune rabbit serum as the primary antiserum and serial sections that were incubated with primary antiserum that had been blocked by preincubation for 16 h at 4° C with antigen (4 µl primary antiserum with 20 µg antigen). During the blocking procedure to prevent proteolysis of antiserum by Cat G or HNE or reduction of free antigen by complex formation between Cat G or HNE and antiproteases in the antiserum, inactive Cat G and HNE were used. Inactive HNE and Cat G were

Table 1. Neutrophil cathepsin G and elastase activity associated with isolated amyloid fibrils*

TYPE	µg HNE	µg Cat G
AL		
#1 (spleen)	0.40±0.10 (2)	0.08±0.02 (2)
#2 (spleen)	0.25±0.05 (2)	0.01±0.01 (2)
AA		
#3 (thyroid)	0.40±0.20 (2)	0.08±0.02 (2)**
#4 (thyroid)	0.07±0.03 (2)	0.33±0.05 (2)**
ATTR		
#5 (heart)	0.60±0.10 (2)	0.38±0.02 (2), 0.05±0.03 (2)†
#6 (heart)	0.40±0.10 (2)	0.37±0.17 (2)
Aß2M		
#7(femoral head)	0.60±0 (2)	0.03 (1)
#8 (heart)	0.08±0.02 (2)	0.02 (1)
AApo A-1		
#9 (liver)	0.04±0.01 (2)	0.05±0.01 (2)

* Activity is expressed as µg purified enzyme equivalents, mean±1 SE (no. of determinations) per 4 mg of fibrils.

** Activity not reduced by addition of Succinyl-L-alanyl-L-alanyl-L-prolyl-L-valine chloromethyl ketone, an inhibitor specific for HNE (n=2).

† Activity reduced by addition of PMSF.

prepared by incubation with Succinyl-L-alanyl-L-alanyl-L-prolyl-L-valine chloromethyl ketone and PMSF, respectively, followed by dialysis.

RESULTS

Enzyme assays

HNE- and Cat G-like activity was found associated with isolated amyloid fibril deposits from patients with AL, AA, hereditary (ATTR and AApo A-1) and Aß2M amyloidosis (Table 1). Relatively large amounts of Cat G-like activity were found in both samples from patients with

Table 2. Assay for collagenase activity in ß2-microglobulin fibrils and synovial fluid samples using [3H]-fibrillar collagen*

cpm released	n	SAMPLE
132±4	(4)	buffer
177	(1)	1 µg HNE+1 µg Cat G
43	(1)	1 µg HNE+1 µg Cat G + PMSF
3033	(1)†	0.5 mg bacterial collagenase
2989	(1)†	1.0 mg bacterial collagenase
2556	(1)†	1.0 mg bacterial collagenase + PMSF
177±24	(3)	ß2M fibrils (4 mg) (patients #8, 12, 13, one sample from each)
116±21	(3)	ß2M fibrils (4 mg) (patients#8, 12, 13) + PMSF
146±8	(2)	100 ml synovial fluid (patients # 10, 11, one sample from each)
164±3	(2)	100 ml synovial fluid (patients #10, 11) + PMSF

* Expressed as the mean±1 standard error. 0.1 ml of a total 1 ml of supernatant was assessed for radioactivity.

† All of the substrate was solubilized.

Table 3. Summary of immunohistochemical results obtained using antisera to HNE and Cat G

Amyloid type	Patient # and tissue	Antisera to HNE	Antisera to Cat G
AL	#1 - liver	negative	negative
AA	#4 - thyroid	positive	positive
ATTR	#5 - kidney	positive	positive
Aß2M	#8 - knee synovium	positive	positive
AApo A-1	#9 - heart	negative	negative

ATTR and from one patient with AA amyloidosis. The amount of Cat G activity ranged from 5-571% of the HNE-like activity associated with the same fibril preparation; the mean relative activity of Cat G as compared with HNE was 103±60% (n=9) (mean±SE). The Cat G-like activity was 87% inhibited by PMSF, an inhibitor of serine proteases, a category that includes both HNE and Cat G. The Cat G activity was not reduced by the presence of Succinyl-L-alanyl-L-alanyl-L-prolyl-L-valine chloromethyl ketone, a specific inhibitor of HNE and porcine pancreatic elastase [8]. HNE- and Cat G-like activity was also found with amyloid fibrils from the CBA/J mouse model (0.4 and 0.2 μg, respectively).

Aß2M fibril samples were examined for collagenolytic activity (Table 2). [^{3}H]-fibrillar collagen was completely solubilized by 0.5 μg of bacterial collagenase, suggesting a sensitivity to less than 0.02 μg bacterial collagenase equivalents. The small amount of collagenolytic activity associated with the added HNE + Cat G or the Aß2M fibril samples was eliminated by the addition of PMSF, suggesting that the activity was due to the presence of HNE and Cat G, serine proteases both inhibited by PMSF.

Immunohistochemistry

In the tissues from patients with AA, ATTR or Aß2M, amyloid deposits, identified in adjacent sections with Congo red, showed a positive reaction to anti-HNE and anti-Cat G, while sections containing AL or AApo A-1 deposits gave a negative reaction (Table 3, Figure 1). Reaction product was not seen when non-immune rabbit serum was used or when the primary antisera to HNE or Cat G were preincubated with HNE or Cat G, respectively.

DISCUSSION

The studies reported here indicate the presence of significant amounts of HNE- and Cat G-like enzyme activity on isolated amyloid fibrils from tissues of patients with 5 different types of systemic amyloidosis (AL, AA, ATTR, Aß2M and AApo A-1, nomenclature in [13]), although amyloid deposits in tissue sections only from patients with AA, ATTR or Aß2M amyloidosis stained positive with antiserum to HNE and Cat G. Measured with a specific substrate, Succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine paranitroanilide, that is rapidly hydrolyzed by Cat G but only slightly (<3%) by HNE [8], the mean relative activity of HNE and Cat G associated with the fibrils was similar. HNE and Cat G are both found in the azurophilic granules of human

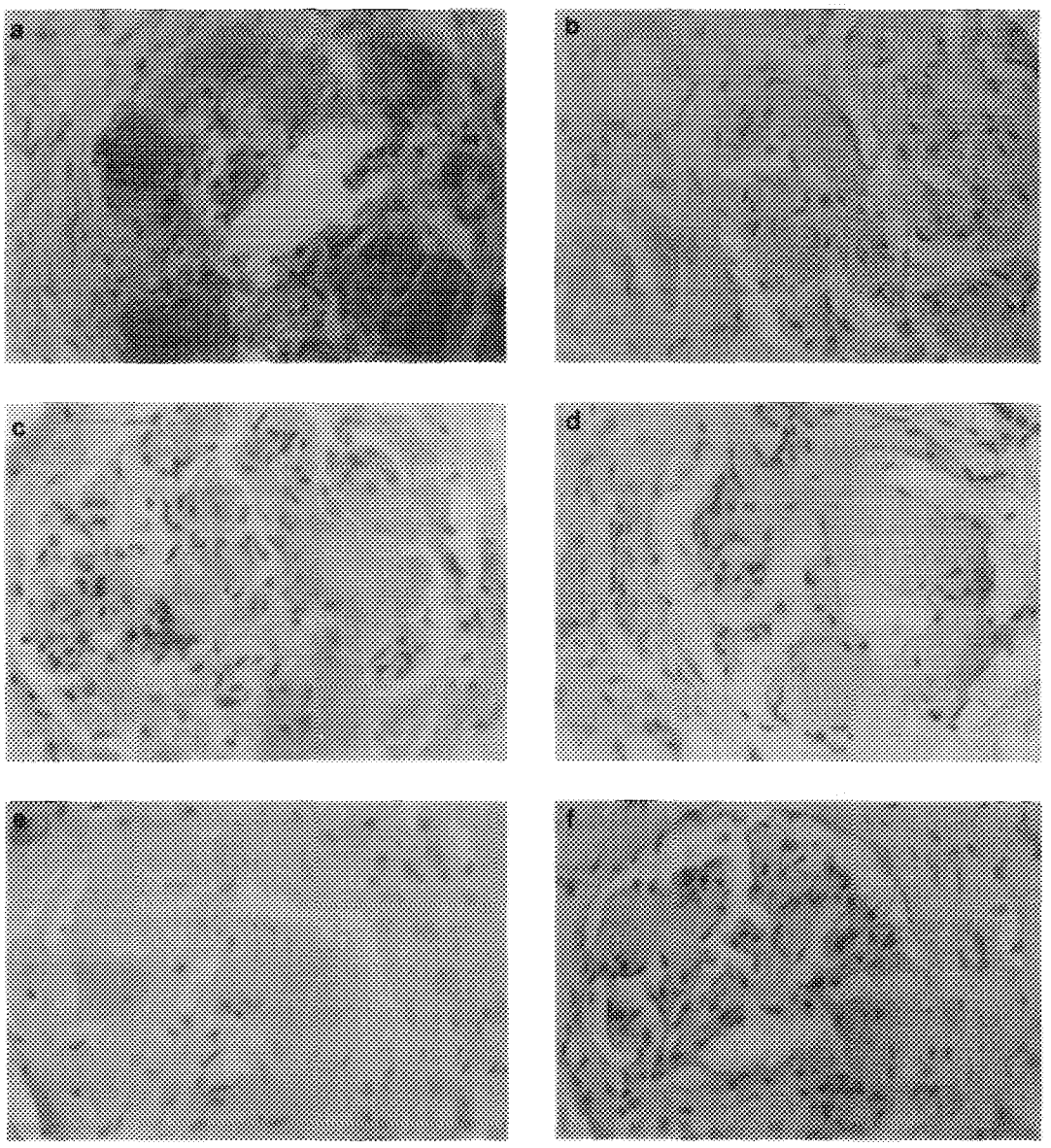

Figure 1. Immunohistochemical staining of ATTR amyloid deposits with anti-HNE and anti-Cat G. As described in Methods, sections of kidney tissue (patient #5), with amyloid deposits identified on adjacent sections with Congo red, were incubated with primary rabbit antisera to HNE or Cat G and visualized by using an avidin-biotin complex method. **a**, Congo red and hematoxylin staining. Amyloid deposits stain with Congo red. **b**, Normal rabbit serum as the primary antiserum. **c**, Anti-HNE. Amyloid deposits show light brown staining, whereas heavy reaction product is located on neutrophils. **d**, anti-HNE that has been preincubated with 20 μg of HNE. **e**, Anti-Cat G. Light brown staining is seen on amyloid deposits. **f**, Anti-Cat G that has been preincubated with 20 μg of Cat G. **b,d,f** are controls and reaction is negative on amyloid deposits. Magnification: all 80 x. Reproduction of Figure 1 in color was made possible by the Extramural Grant Program of Baxter Healthcare Corporation, McGaw Park, Illinois, USA.

neutrophils and, to a lesser extent, in monocytes, and can be copurified in approximately equal quantities from neutrophils [14,15]. The Cat G-like activity was not due to the HNE associated with the fibrils, since an HNE-specific chloromethyl ketone compound that effectively inhibits

HNE irreversibly, did not alter the amount of Cat G-like activity [8]. As expected the Cat G-like activity was inhibited 87% by the addition of PMSF, an inhibitor of serine proteases. The finding of HNE- and Cat G-like enzymatic activity associated with amyloid fibrils also extends to the CBA/J mouse model of casein-induced AA amyloidosis.

The presence of enzymatically active HNE and Cat G associated with amyloid fibrils, after the preparation of these fibrils from tissue is surprising. First, losses of HNE occur during each washing step [5]. It is also likely that Cat G losses occur during the washing step. Second, it is surprising that, in vivo or after extraction of the fibrils, the enzymes have not been inactivated irreversibly by the presence of a potent inhibitor, alpha-1-protease inhibitor. Alpha-1-protease inhibitor is found in extravascular compartments of major organs [16]. We considered the possibility that much of the HNE and Cat G may have come from neutrophils, present in the tissue and released by the extraction procedure. However, examination of sections of the tissue from which the fibrils were extracted, revealed the presence of few neutrophils. Based on a content of HNE in neutrophils of 6 pg per cell, the ATTR specimens would have to contain as many as 100,000 neutrophils in order to release the amount of measured HNE [17]. There would be significant losses from mixing of HNE and Cat G with protease inhibitors present in the tissue and blood vessels and further losses due to washing [5].

Fibrillogenesis of amyloid proteins may involve a number of factors including: amino acid sequence variability which may alter the protein stability when in contact with proteases; an amyloid enhancing factor known to be present in all amyloidotic tissues; associated matrix molecules such as glycosaminoglycans; a high concentration of amyloid protein (β_2M, serum amyloid A); and other factors such as time. One might propose the following hypothesis for the sequence of events in the formation of some amyloid deposits. Inflammation brings white blood cells to a tissue site. As some of them lyse, releasing lysosomal enzymes, a microenvironment similar to that in the lysosome is created with an acidic pH of 5 or lower. Amyloid proteins may also be engulfed in phagolysosomes of the white blood cells and interact there with HNE and Cat G. Others have postulated that the initiation of the early events in fibril formation may occur in lysosomes [1,18]. Amyloid proteins are present, perhaps, at higher concentrations than normal, if they are acute phase reactants, i.e. serum amyloid A. Mutant precursor proteins may also be present. Either factor may lead to higher levels of degradation, since clearance mechanisms for achieving homeostasis will be upregulated and mutant proteins may be susceptible to abnormal degradation that would generate amyloidogenic fragments. HNE and Cat G may become associated with the fibrils during a proteolytic processing step of precursor material as suggested by earlier studies [3,4]. Small peptides which are ß sheet forming precipitate in tissues creating a nidus. Tissue deposits increase by deposition of fragments and full length precursor proteins. The inability of HNE and Cat G, even in combination and in large amounts, to visually digest fibrils was shown when we added 0.5 mg of HNE and 1 mg of Cat G to 4 mg of ATTR fibrils (patient #5) and incubated at 37° C (data not presented)

With regard to the presence of activated collagenase in synovial fluid or Aβ_2M fibril samples from patients with β_2M amyloidosis, we found no activity able to solubilize fibrillar collagen. Investigators have found collagenolytic activity present in synovial fluid from patients

with rheumatoid arthritis [19]. We did not assess latent collagenolytic activity. The fibrillar collagen used as a substrate here was not derived from solubilized collagen and allows for measurement of collagenolytic activity by the presence of radioactivity released into the solution.

In conclusion we propose that the proteases HNE and Cat G, found in the azurophilic granules of the neutrophil and the monocyte, may become associated with the amyloid fibrils before or during formation of the fibrils. This may occur in an extracellular inflammatory microenvironment at an acidic pH or in a phagolysosome and may play a role in the formation of the fibrils. The effect of these proteases on the kinetics of formation of amyloid deposits is still not known.

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