PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 181, 211–214 (1986)

The Association of an Elastase with Amyloid Fibrils[1] (42242)

MARTHA SKINNER,*† PHILLIP STONE,‡ TSURANOBU SHIRAHAMA,*† LAWREEN HELLER CONNORS,† JAMES CALORE,‡ AND ALAN S. COHEN*†

Thorndike Memorial Laboratory and Division of Medicine, Boston City Hospital, Boston, Massachusetts 02118, †Arthritis Center and ‡Biochemistry Department, Boston University School of Medicine, Boston, Massachusetts 02118

Abstract. The fibrils of all systemic forms of amyloid (primary, AL; secondary, AA; and hereditary, AF) that had been isolated by the water extraction procedure demonstrated elastolytic enzyme activity when examined in a specific assay using tritiated elastin. The source of this fibril-bound enzyme activity was consistent with human neutrophil elastase (HNE), since it was readily extracted by high salt solutions and inhibited by an elastase-specific chloromethyl ketone inhibitor, human α-1-protease inhibitor or by an antibody specific for HNE. The presence of an elastase on the amyloid fibril may suggest physiologic mechanisms of amyloid precursor protein degradation.

The mechanism by which certain serum protein precursors are processed into amyloid fibrils remains unknown; however, proteolytic enzymes are thought to play a role. The serum protein precursor varies in each type of systemic amyloidosis and some proteolysis (processing) is evident when the precursor protein is compared to the final amyloid fibril product (1). This processing may represent partial proteolysis of a precursor or the inability of normal proteolysis to occur.

Some degree of proteolysis is suggested in the primary (AL) form of amyloid where fibrils are composed of various sized fragments of immunoglobulin light chains (2–4). In some cases only the variable half is present, in others the variable half as well as a portion of the constant half composes the amyloid fibril. In a few cases the fibril represents the whole light chain. Some proteolysis is evident in the secondary (AA) amyloid type where the fibrils are nearly uniformly an 8000-Da portion (AA) of the 12,000-Da serum amyloid A (SAA) protein (5–8). In the hereditary (AF) amyloid type, the precursor protein is a whole prealbumin molecule, where no proteolysis to form amyloid fibrils is apparent (9–11).

A role for proteolytic enzymes closely associated with amyloid fibrils has been suggested by the appearance of fibril degradation in the amyloid–agarose plate test (12, 13). In the course of our investigation of the degradation of amyloid fibrils, we found a serine protease, consistent with human neutrophil elastase (HNE), associated with all forms of amyloid fibril isolates. In this paper we report that an elastase appears to be intimately associated with all amyloid fibril types as they are isolated from the tissue and may play a role in fibril formation from precursor proteins.

Methods. *Preparation of amyloid fibrils.* AA, AL, and AF amyloid fibrils were extracted and purified from amyloid-rich tissues according to methods previously described (14, 15). AA fibrils were obtained from the liver of a patient with ankylosing spondylitis. AL fibrils were obtained from the liver or spleen of several patients with primary amyloidosis. AF fibrils were obtained from the thyroid of a patient of Swedish origin with hereditary (prealbumin) amyloidosis.

Elastase assay. Fibrils were assayed for elastin-solubilizing activity. The fibrils were incubated at 37°C for 20 ± 4 hr in the presence of washed ^{3}H calf ligamentum nuchae elastin in 4 ml buffer (0.05 *M* Tris, pH 7.6, 0.14 *M* NaCl, 0.05% NaN_3) (16, 17). At the end of the incubation the samples were centrifuged and the supernatants filtered. The amount of elastin solubilized was calculated from the level of radioactivity present corrected for the background level of radioactivity released from

[1] Presented in abstract at the IV International Symposium on Amyloidosis, Harriman, N.Y., November 10, 1984.

0037-9727/86 $1.50

^{3}H elastin in buffer only. By comparing the tubes containing ^{3}H elastin and standard amounts of a highly purified and enzymatically active HNE prepared from human sputum (18, 19), the levels of elastolytic activity were expressed as HNE equivalents. Assays were performed on 4 mg of AA, AL, or AF fibrils either alone or modified as follows: with an elastase-specific inhibitor, succinyl-(L-alanyl)$_2$-prolyl-valyl-chloromethyl ketone (CMK) (20); with α-1-protease inhibitor; or with an IgG fraction of either rabbit anti-human neutrophil elastase antibody or porcine pancreatic elastase antibody. In addition, fibril preparations were assayed after boiling for 10 min in 0.1 *M* Tris, pH 7.6.

Elastase extraction with salt solutions. The ability of high and low salt concentrations to extract elastolytic activity from AA amyloid fibrils was examined. Four-milligram portions of AA fibrils were incubated overnight at 4°C with buffer containing either 0.6 or 0.14 *M* NaCl. After centrifugation the supernatants of the 0.14 NaCl extraction were adjusted to 0.6 *M* NaCl before the assay procedure.

Results. In the tritiated elastin assay for enzymatically active human neutrophil elastase, all three fibril types (AL, AA, and AF) had significant amounts of elastolytic activity ranging from 150 to 1383 ng HNE equivalents per 4 mg amyloid fibrils (Table I). The AL fibrils consistently had less elastolytic activity than the AA or AF fibrils. Six additional AL preparations were examined in duplicate and showed varying amounts of HNE equivalents (range 0–790 ng). Elastolytic activity was inhibited by the presence of CMK, an antibody to HNE, human α-1-protease inhibitor, and by boiling the fibrils prior to assay. No significant inhibition of the AA sample activity (998 ng HNE equivalents) was found when a rabbit IgG fraction of porcine pancreatic elastase antibody shown to have no anti-human neutrophil elastase activity was used as a control antiserum. Lack of sufficient quantities of AF fibrils precluded three of the determinations.

Elastolytic activity could be extracted from the AA amyloid fibrils both by high and low salt solutions; however, five times as much elastolytic activity was extracted from AA fibrils with buffer containing 0.6 *M* NaCl than with 0.14 *M* NaCl (Table II). Less elastolytic activity remained on the fibrils extracted with the buffer containing 0.6 *M* NaCl than with 0.14 *M* NaCl.

Discussion. Isolated fibrils of the three systemic forms of amyloidosis were examined for elastolytic activity in a specific assay using tritiated elastin. Significant amounts were present. The dramatic inhibition of this enzyme activity by an elastase-specific chloromethyl ketone inhibitor, an antibody to human neutrophil elastase, and by human α-1-protease inhibitor strongly supports the identification of this enzyme on the fibril as an elastase which is consistent with HNE. Further evidence supporting the identity of an elastase on the fibril was the fact that more elastolytic activity could be extracted from the fibrils with solutions of higher salt concentration than with lower salt concentration (21). The amount of elastolytic activity on the AA and AF fibrils was consistently high. However, the amount of elastolytic activity on the AL-type amyloid fibrils varied widely in six preparations tested. Since the fibril isolation involves repeated washings of the fibrils with a low salt solution (0.14 *M* NaCl) and our studies showed that elastolytic activity could be removed by salt solutions, it is very likely that some of the elastase was removed

TABLE I. ELASTASE ACTIVITY ON AMYLOID FIBRILS

Amyloid fibrils (4 mg)	ng HNE equivalents ± SE (*n*)		
	AA	AL	AF
Alone	1383 ± 228 (2)	150 ± 11 (3)	1029
Plus elastase-specific chloromethyl ketone	33 ± 3 (2)	15 ± 4 (3)	62
Plus HNE antibody (rabbit IgG fraction)	49	49 ± 15 (2)	ND
Plus α-1-protease inhibitor	22 ± 1 (2)	28 ± 7 (2)	ND
Boiled	20 ± 3 (2)	23 ± 8 (2)	ND

Note. ND = not determined. *n* = Number of replicate samples in same assay.

TABLE II. SALT EXTRACTABILITY OF ELASTOLYTIC ACTIVITY ON AA FIBRILS

Salt concentration of extraction buffer	Supernatant activity[a] (ng)	Activity remaining on fibrils[a] (ng)
0.6 M NaCl	622 ± 19	591 ± 20
0.14 M NaCl	122 ± 11	862 ± 32

[a] Mean ± SE (n = 3) ng of HNE equivalents.

from the fibrils. Perhaps the elastase associated with the AL fibrils was more susceptible to removal by saline washes than that associated with the other fibril types.

Proteolytic enzymes have been involved in the processing of precursor protein into AL and AA fibrils. In separate studies, Glenner (2) and Shirahama (3) have shown that homogenous immunoglobulins (Bence–Jones protein) could be digested by pepsin to form fibrillar material resembling amyloid fibrils. This did not occur with all Bence–Jones proteins, although there is no unique precursor immunoglobulin type for AL amyloid fibrils. In addition, AL fibril molecular weight has varied from 11,000 to 23,000 Da suggesting that in many cases proteolysis of the light chain occurs prior to its processing into an amyloid fibril (4). Secondary amyloid fibril proteins have varied little in size or primary sequence. They represent an 8000-Da molecule which is derived from the 12,000-Da serum amyloid A precursor, after cleavage of the C-terminal portion (5, 6). Studies have shown that degradation of the SAA protein into AA-sized fragments can be accomplished with a monocytic elastase (6, 7) or with human neutrophil elastase (8). The heredofamilal amyloid fibrils are composed of the monomeric prealbumin subunit of 14,000 Da with no apparent degradation occurring prior to processing. In this form of amyloid a variant primary structure is believed responsible for its selective fibril formation (9).

One may speculate that an elastase may play a role in the normal proteolysis of proteins which are amyloid precursors. This mechanism may be slowed or blocked in amyloidosis because of an amino acid substitution within the precursor protein. It is well known that valine is a primary cleavage site for HNE (20). Although structural studies on amyloid proteins are limited at present, in two cases a valine substitution is known to have occurred. In the variant prealbumin precursor protein which forms AF amyloid fibrils, a valine has been replaced by a methionine at position 30 (9–11). In the murine form of AA amyloid, the amyloidogenic SAA precursor protein has a valine replaced by a phenylalanine at position 6 (22, 23). These positions may represent important initial cleavage sites before other valines within the proteins can undergo cleavage. When cleavage does not occur at these sites, other less available sites may be masked by a tight protein conformation or by bound substances on the fibril such as amyloid P component (15).

The present study has demonstrated the close association of an elastase with the amyloid fibril. It is present on amyloid fibrils of all types and in significant amounts. Although the role of specific enzymes in fibril formation and/or degradation is not yet known, its presence suggests it may participate in physiologic mechanisms of precursor protein degradation.

The authors gratefully acknowledge the expert assistance of Ms. Maureen Haley in the preparation of this manuscript. Supported by grants from the U.S. Public Health Service, NIAMDD (AM 04599 and AM 07014), from the General Clinical Research Centers Branch of the Division of Research Resources, National Institutes of Health (RR 533), Multipurpose Arthritis Center, National Institutes of Health (AM 20613), National Institutes of Health (HL 19717, HL 25229), and the Arthritis Foundation.

1. Benditt EP, Cohen AS, Costa PP, Franklin EC, Glenner GG, Husby G, Mandema E, Natvig JB, Osserman EF, Sohar E, Weglius O, Westermark P. Nomenclature. In: Glenner GG, Costa PP, Freitas AF, eds. Amyloid and Amyloidosis. Amsterdam, Excerpta Medica, ppxi–xii, 1980.
2. Glenner GG, Ein D, Eanes ED, Bladen HA, Terry W, Page DL. Creation of "amyloid" fibrils from Bence Jones proteins in vitro. Science (Washington, DC) **174:** 712–714, 1971.
3. Shirahama T, Benson MD, Cohen AS, Tanaka A. Fibrillar assemblage of variable segments of immunoglobulin light chains: An electron microscopic study. J Immunol **110:**21–30, 1973.
4. Glenner GG. Amyloid deposits and amyloidosis: The beta-fibrilloses. N Engl J Med **302:**1283–1292, 1333–1343, 1980.
5. Sipe JD, Ignaczak TF, Pollock PS, Glenner GG. Amyloid fibril protein AA: Purification and properties of the antigenetically related serum component as de-

termined by solid phase radioimmunoassay. J Immunol **116**:1151–1156, 1976.
6. Lavie G, Zucker-Franklin D, Franklin EC. Degradation of serum amyloid A protein by surface associated enzymes of human blood monocytes. J Exp Med **148**:1020–1031, 1978.
7. Lavie G, Zucker-Franklin D, Franklin EC. Elastase-type proteases on the surface of human blood monocytes: Possible role in amyloid function. J Immunol **125**:175–180, 1980.
8. Silverman SL, Cathcart ES, Skinner M, Cohen AS. The degradation of serum amyloid A protein by activated polymorphonuclear leucocytes: Participation of granulocytic elastase. Immunology **46**:737–744, 1982.
9. Dwulet FE, Benson MD. Primary structure of an amyloid prealbumin and its plasma precursor in a heredofamilial polyneuropathy of Swedish origin. Proc Natl Acad Sci USA **81**:694–698, 1984.
10. Saraiva MJ, Costa PP, Goodman DS. Studies on plasma transthyretin (prealbumin) in familial amyloidotic polyneuropathy, Portugese type. J Lab Clin Med **102**:590–603, 1983.
11. Tawara S, Nakazato M, Kangawa K, Matsuo H, Araki S. Identification of amyloid prealbumin variant in familial amyloidotic polyneuropathy (Japanese type). Biochem Biophys Res Commun **116**:880–888, 1983.
12. Kedar I, Sohar E, Ravid M. Degradation of amyloid by a serum component and inhibition of degradation. J Lab Clin Med **99**:693–700, 1982.
13. Skinner M, Schneller SI, Connors LH, Kagan HM, Stone PJ, Shirahama T, Sipe JD, O'Connor C, Calore JD, Franzblau C, Cohen AS. Observations on the amyloid-degrading activity of serum and its relationship to human neutrophil elastase. Trans Assoc Amer Physicians **96**:437–443, 1983.
14. Pras M, Schubert M, Zucker-Franklin D, Rimon A, Franklin EC. The characterization of soluble amyloid prepared in water. J Clin Invest **47**:924–933, 1968.
15. Skinner M, Shirahama T, Cohen AS, Deal CL. The association of amyloid P-component (AP) with the amyloid fibril: An updated method for amyloid fibril isolation. Prep Biochem **12**:461–476, 1983.
16. Stone PJ, Calore JD, Snider GL, Franzblau C. Role of alpha-macroglobulin elastase complexes in the pathogenesis of elastase-induced emphysema in hamsters. J Clin Invest **69**:920–931, 1982.
17. Stone PJ, Franzblau C, Kagan KM. Proteolysis of insoluble elastin. In: Cunningham LW, Frederiksen DW, eds. Methods in Enzymology. New York, Academic Press, Vol 82:pp588–605, 1982.
18. Martodam RR, Baugh RJ, Twumasi DY, Liener IE. A rapid procedure for the large scale purification of elastase and cathepsin G from human sputum. Prep Biochem **9**:15–31, 1979.
19. Snider GL, Lucey EC, Christensen TG, Stone PJ, Calore JD, Catanese A, Franzblau C. Emphysema and bronchial secretory cell metaplasia induced in hamsters by human neutrophil products. Amer Rev Respir Dis **129**:155–160, 1984.
20. Powers JC, Gupton BF, Harley AD, Nishino N, Whitley RJ. Specificity of porcine pancreatic elastase, human leukocyte elastase and cathespin G. inhibition with peptide chloromethyl ketones. Biochim Biophys Acta **485**:156–166, 1977.
21. Baugh RJ, Travis J. Human leukocyte granule elastase: Rapid isolation and characterization. Biochemistry **15**: 836–841, 1976.
22. Hoffman JS, Ericsson LH, Eriksen N, Walsh KA, Benditt EP. Murine tissue amyloid protein AA: NH_2-terminal sequence identity with only one of two serum amyloid protein (apo SAA) gene products. J Exp Med **159**:641–646, 1984.
23. Skinner M, Shirahama T, Benson MD, Cohen AS. Murine amyloid protein AA in casein induced experimental amyloidosis inhibitor. Lab Invest **36**:420–427, 1977.

Received June 4, 1985. P.S.E.B.M. 1986, Vol. 181.
Accepted October 8, 1985.