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To the editor:

Two types of amyloid in a single heart

Amyloidosis is a heterogeneous group of diseases, in which amyloidogenic precursor proteins misfold and adopt a β-pleated sheet conformation.[1] Several proteins can form amyloid fibrils in vivo including transthyretin,[2] apolipoprotein A-I and A-II, lysozyme, fibrinogen, serum amyloid A protein and immunoglobulin light chains, but "cross-fibril" seeding appears to be rare such that 2 types of amyloid are rarely identified in the same individual.[3] Congo red (CR) staining with apple green birefringence under polarized light is used to confirm the presence of amyloid,[4] whereas immunohistochemistry, staining the biopsy tissue with a panel of monospecific antibodies against known amyloidogenic proteins, is the technique most widely used to characterize the amyloid fibril protein, but it is flawed.[5] Proteomic analysis involves proteolytic cleavage of proteins within microdissected amyloidotic tissue and identification by mass spectometry.[6] This additional tool is being increasingly used in conjunction with immunohistochemistry to identify the amyloid fibril protein.

We describe a case in which 2 amyloid fibril proteins were isolated in an individual patient both by immunohistochemistry and by proteomic analysis. An 81-year-old man was referred to our center with a 6- to 7-month history of exertional dyspnea and New York Heart Association class II symptoms. Baseline investigations included an echocardiogram showing characteristic features of cardiac amyloidosis with a thickened interventricular wall of 18 mm, moderate diastolic dysfunction, and preserved left ventricular ejection fraction of 58% accompanied by elevated serum cardiac biomarkers (*N*-terminal fragment brain natriuretic peptide 1966 ng/L and troponin T 0.09 μg/mL). ^{123}I-labeled serum amyloid P component scintigraphy did not show visceral amyloid deposits,[7] but ^{99m}Tc-dicarboxypropane diphosphonate (^{99m}Tc-DPD) scintigraphy showed abnormal (Perugini grade 2) cardiac uptake, typical of cardiac transthyretin amyloidosis[8] and unusual in light chain (AL) amyloidosis.[9] The κ-serum free light chain concentration was 13.1 mg/L, λ was 644 mg/L, and λ BJP was present. A bone marrow biopsy showed 6% plasma cells and immunophenotyping isolated a CD19$^-$, CD56$^+$, CD27$^+$ plasma cell clone. Sequencing of the transthyretin gene was wild-type. The differential diagnosis was between wild-type cardiac transthyretin (ATTR) amyloidosis (senile systemic amyloidosis) and cardiac AL amyloidosis, the management and prognosis of which differ substantially. A cardiac biopsy was undertaken to differentiate between these diagnoses. Figure 1 shows CR and immunohistochemical staining of the specimen using antibodies to λ light chains and transthyretin, and the results of proteomic analysis. Interestingly, there were 2 distinct patterns of amyloid within the same specimen: 1 showed honeycomb morphology, lighter CR staining, and stained with antibody against transthyretin, but not λ light chains, and the other showed more diffuse, but darker CR staining, and stained with antibody against λ light chains, but not transthyretin. The 2 distinct areas were separately captured by laser microdissection and analyzed by tandem mass spectrometry. Amyloid was identified by its "signature proteins" in both samples, but in 1 there was abundant transthyretin with low level λ light chain and in the other there was abundant λ light chain without transthyretin (Figure 1D), thus confirming the immunohistochemistry results of 2 types of amyloid in the same heart. Our patient is due to receive chemotherapy for AL amyloidosis shortly.

In summary, the exceptionally rare occurrence of 2 different amyloid fibril proteins was suggested by immunohistochemistry in this patient. The coexistence of 2 amyloid types in the same heart was confirmed by laser capture microdissection and proteomic analysis of 2 distinct areas.

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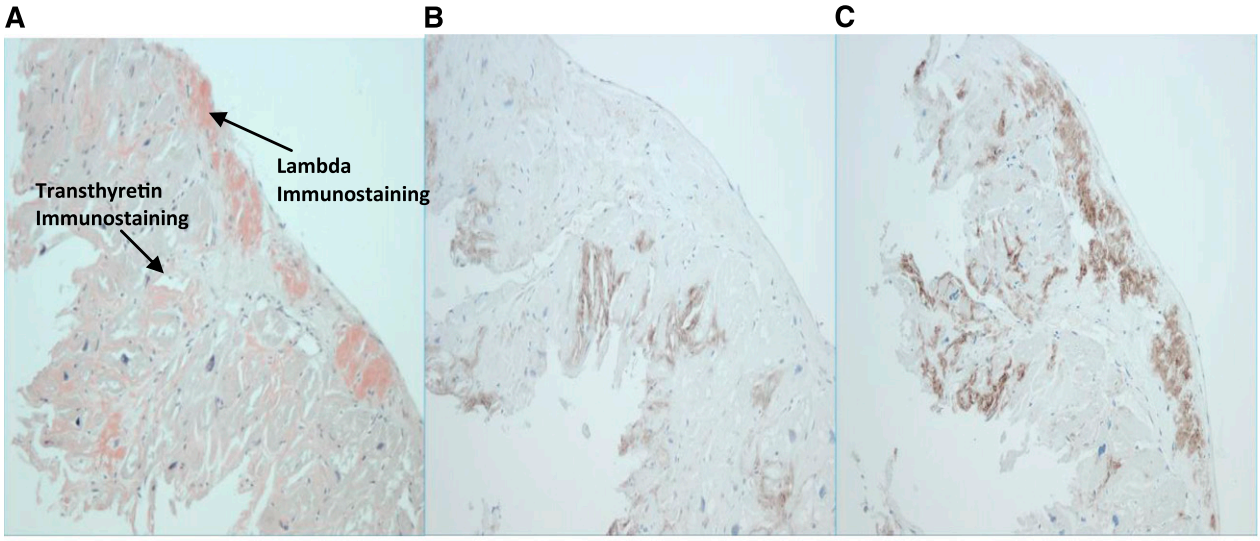

D

Probability Legend:
over 95%
80% to 94%
50% to 79%
20% to 49%
0% to 19%

Bio View:
Identified Proteins (45)
Including 0 Decoys

#	Visible?	Starred?		Accession Number	Molecular Weight	Protein Grouping Ambiguity	Sample X	Sample Y
1	☑	★	Apolipoprotein A-IV OS=Homo sapiens GN=APOA4 PE=1 SV=3	APOA4_HUMAN	45 kDa		14	2
2	☑	★	Apolipoprotein E OS=Homo sapiens GN=APOE PE=1 SV=1	APOE_HUMAN	36 kDa		11	3
3	☑	★	Ig lambda-2 chain C regions OS=Homo sapiens GN=IGLC2 PE=1 SV=1	LAC2_HUMAN (+1)	11 kDa		7	4
4	☑	★	Serum amyloid P-component OS=Homo sapiens GN=APCS PE=1 SV=2	SAMP_HUMAN	25 kDa		6	4
5	☑	★	Transthyretin OS=Homo sapiens GN=TTR PE=1 SV=1	TTHY_HUMAN	16 kDa			10
6	☑	★	Apolipoprotein A-I OS=Homo sapiens GN=APOA1 PE=1 SV=1	APOA1_HUMAN	31 kDa		2	2
7	☑	★	Ig kappa chain C region OS=Homo sapiens GN=IGKC PE=1 SV=1	IGKC_HUMAN	12 kDa		2	1

E

GQPKAAPSVT LFPPSSEELQ ANKATLVCLI SDFYPGAVTV AWKADSSPVK
AGVETTTPSK QSNNKYAASS YLSLTPEQWK SHRSYSCQVT HEGSTVEKTV
APTECS

F

MASHRLLLLC LAGLVFVSEA GPTGTGESKC PLMVKVLDAV RGSPAINVAV
HVFRKAADDT WEPFASGKTS ESGELHGLTT EEEFVEGIYK VEIDTKSYWK
ALGISPFHEH AEVVFTANDS GPRRYTIAAL LSPYSYSTTA VVTNPKE

Figure 1. Immunohistochemical staining and proteomic analysis showing 2 distinct patterns of amyloid within the cardiac biopsy. (A) Congo red staining of the myocardium. There was an area of dark Congo red staining that also stained with an antibody against λ light chain, but not transthyretin, and an area of lighter Congo red staining that stained with an antibody against transthyretin, but not λ light chain. (B) Transthyretin immunostaining confirming the presence of transthyretin. (C) Immunostaining with an antibody against λ light chain. (D) Proteomic analysis illustrating spectral counts and peptide coverage. Both samples showed the amyloid signature proteins apolipoprotein A-IV, serum amyloid P component, and apolipoprotein. (E) Sample X (left) shows abundant λ light chain (7 spectra, 57% protein coverage in yellow highlight [E]) without any transthyretin and sample Y (right) shows abundant transthyretin (10 spectra, 41% protein coverage in yellow highlight [F]) and some λ light chain (4 spectra, 14% protein coverage). The protein identification probability was >95% in each case.

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To the editor:

A germ line mutation in cathepsin B points toward a role in asparaginase pharmacokinetics

L-Asparaginase (ASNase) is a key component of protocols used to treat acute lymphoblastic leukemia (ALL). Poorly understood interpatient differences in ASNase pharmacokinetics demand therapeutic drug monitoring to prevent patients from receiving an inadequate dose.[1] Although it is unclear whether there is a causal relation between elevated ASNase levels and toxicities, underexposure compromises therapeutic benefits. A recent report demonstrated that lysosomal proteases degrade ASNase in vitro.[2] However, to which extent these proteases contribute to ASNase clearance in patients remains unclear. Here we link a strongly prolonged ASNase turnover to a germ line mutation in the gene encoding cathepsin B.

A pediatric patient, treated for common B-cell progenitor ALL, developed encephalopathy associated with hyperammonemia (354 μmol/L) after the sixth dose of Erwinase. She required plasmapheresis to reduce serum ammonia levels. Serum analysis revealed abnormally high concentrations of Erwinase at the time of plasmapheresis (2167 IU/mL, 2 days after the last dose). After recovery, Erwinase treatment was restarted under therapeutic drug monitoring. This revealed a strongly increased half-life of Erwinase (Figure 1A), which could explain the high Erwinase serum concentrations (see supplemental Case Report for detailed information; available on the *Blood* Web site).

We hypothesized that a defect in one of the lysosomal proteases, previously reported to be capable of degrading asparaginase in vitro,[2] might be responsible for the prolonged half-life observed in this patient. Sequencing of DNA isolated from peripheral blood mononuclear cells (PBMCs) and from buccal cells revealed a heterozygous single codon deletion (c.709_711delAAG) in the gene encoding cathepsin B in the germ line of the patient, which is not listed in the Database of Single Nucleotide Polymorphisms (dbSNP; National Center for Biotechnology Information, Bethesda, MD) or in our in-house database containing the exome sequence data of 1154 individuals (Figure 1B). This mutation results in a deletion of a highly conserved lysine residue in the C terminus of the protein (p.K237del), which is predicted[3] to lead to a loss of structural integrity of the protein (supplemental Figure 1A-B).

Cathepsin B is synthesized as a 44-kDa pre-proenzyme that is processed in late endosomes to a 33-kDa active single chain and matured into an active 2-chain form consisting of a 24-kDa heavy chain (and a 27-kDa glycosylated form) and a 5-kDa light chain.[4] We expressed both wild-type and mutant cathepsin B, cloned from RNA extracted from patient PBMCs, in HEK293 cells to follow the maturation process. Biochemical analysis revealed defective maturation of the mutant cathepsin B, which was confirmed by an aberrant subcellular localization (supplemental Figure 1C-F).

To test whether this mutation affects the protease activity, we assessed cathepsin B activity in Epstein-Barr virus (EBV)–immortalized B cells obtained from the patient. Indeed, cathepsin B activity in the B cells obtained from the patient was strongly reduced as compared with B cells from age-matched donors (Figure 1C), indicating that deletion of residue K237 results in a loss of function.

Next, we determined whether the reduced protease activity of the mutant cathepsin B would result in a diminished degradation of ASNase. Therefore, we incubated Erwinase in lysates of cells expressing either wild-type or mutant cathepsin B and analyzed samples taken at the indicated time points for residual ASNase activity (Figure 1D). Mock transfected cells showed limited protease activity and cleared the Erwinase after 20 hours of incubation. Of note, this degradation was inhibited by the addition of a specific cathepsin B inhibitor, CA-074 (color marked data points, Figure 1D), indicating that endogenous cathepsin B is responsible for ASNase degradation in these lysates. Overexpression of the wild-type cathepsin B resulted in a rapid clearance of the Erwinase from the lysate, which again was fully inhibited by the addition of the cathepsin B inhibitor CA-074. Expression of the K237del mutant cathepsin B protein was still capable of degrading ASNase, but the rate of clearance was significantly reduced ($P < .05$) in comparison with the wild-type protein, consistent with the delayed ASNase clearance observed in this patient. Western blot analysis of asparaginase incubated in these cell lysates confirmed that degradation of asparaginase protein rather than the inhibition of enzymatic activity causes the decrease in asparaginase activity that we measured in the previous assay (supplemental Figure 1G-H).

It is unknown where degradation of ASNase occurs. Low levels of cathepsin B activity are detected in human serum (data not shown), but these amounts are insufficient to degrade *Erwinase* or *Escherichia coli* ASNase in vitro (supplemental Figure 1). Instead, it appears that cathepsin B–mediated degradation of ASNase occurs intracellularly, after being removed from the blood by phagocytic cells, which is consistent with the fact that pegylated forms of this protein show an increased serum half-life.[5]

Current knowledge of factors influencing ASNase pharmacokinetics is limited.[5,6] Only the presence of inhibitory antibodies that bind asparaginase is known to significantly shorten the half-life of ASNase.[7] In our patient, we found no evidence of an immune response targeting the ASNase. Our observations support the