Cross β-Sheet Conformation of Keratin 8 Is a Specific Feature of Mallory–Denk Bodies Compared With Other Hepatocyte Inclusions

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BACKGROUND & AIMS: Mallory–Denk bodies (MDBs) are cytoplasmic protein aggregates in hepatocytes in steatohepatitis and other liver diseases. We investigated the molecular structure of keratin 8 (K8) and 18 (K18), sequestosome 1/p62, and ubiquitin, which are the major constituents of MDBs, to investigate their formation and role in disease pathogenesis. **METHODS:** Luminescent conjugated oligothiophenes (LCOs), h-HTAA, and p-FTAA are fluorescent amyloid ligands that specifically bind proteins with cross β-sheet conformation. We used LCOs to investigate conformational changes in MDBs in situ in human and murine livers as well as in transfection studies. **RESULTS:** LCO analysis showed cross β-sheet conformation in human MDBs from patients with alcoholic and nonalcoholic steatohepatitis or hepatocellular carcinoma, but not in intracellular hyaline bodies, α_1-antitrypsin deficiency, or ground-glass inclusions. LCOs bound to MDBs induced by 3,5-diethoxycarbonyl-1,4-dihydrocollidine feeding of mice at all developmental stages. CHO-K1 cells transfected with various combinations of *SQSTM1/p62*, *ubi*, and *Krt8/Krt18* showed that K8 was more likely to have cross β-sheet conformation than K18, whereas p62 never had cross β-sheet conformation. The different conformational properties of K8 and K18 were also shown by circular dichroism analysis. **CONCLUSIONS: K8 can undergo conformational changes from predominantly α-helical to cross β-sheet, which would allow it to form MDBs. These findings might account for the observation that *krt8*$^{-/-}$ mice do not form MDBs, whereas its excess facilitates MDB formation. LCOs might be used in diagnosis of liver disorders; they can be applied to formalin-fixed, paraffin-embedded tissues to characterize protein aggregates in liver cells.**

Keywords: Liver Disease; Protein Aggregation; Keratin; p62; NASH.

BASIC AND TRANSLATIONAL LIVER

Protein aggregates consisting of misfolded proteins are hallmarks of several chronic diseases, such as neurofibrillary tangles and amyloid plaques in Alzheimer's disease,[1] Lewy bodies in Parkinson's disease,[2] and Mallory–Denk bodies (MDBs) in steatohepatitis.[3] Alterations of hepatocytic keratin intermediate filaments (IFs) with MDB formation are characteristic features of alcoholic steatohepatitis (ASH) and nonalcoholic steatohepatitis (NASH).[4,5] MDB formation also results from drug intoxication and chronic cholestasis, particularly in primary biliary cirrhosis. Furthermore, MDBs might be present in Wilson's disease, idiopathic copper toxicosis, Indian childhood cirrhosis, α_1-antitrypsin (α1-AT) deficiency, hepatocellular neoplasms, and after intestinal bypass surgery.[4–6]

The biological role of MDBs in steatohepatitis is still largely unknown, but they are generally associated with severity of the disease. Although there is detailed information about the primary composition of MDBs, little is known about their molecular structure and whether a specific molecular structure would have functional implications. The filaments of MDBs differ from "classic" IFs in that they have diameters ranging from 3 to 24 nm (vs the typically 10-nm sized IFs) and are coated with amorphous material.[3,7,8] The difference was further shown by monoclonal antibodies recognizing conformation-dependent epitopes on keratins in MDBs.[9] Moreover, pressure-tuning infrared spectroscopy revealed that the typical α-helical signature of native keratin was reduced whereas β-sheet structure increased in the cytoskeleton-enriched fraction of MDB-containing hepatocytes of griseofulvin-treated mice.[10] Furthermore, Kachi et al reported an increase in the relative amounts of β-sheet in isolated mouse and human MDBs using infrared spectroscopy.[11] Although the method applied in these studies did not allow in situ investigation of protein aggregates, it was the first evidence that MDBs differ from IFs on the molecular structural level.

Recently, a relationship between MDBs and intracellular hyaline bodies (IHBs) was reported. IHBs, like MDBs, contain ubiquitin and p62, but keratin is not detectable.[12] MDBs and IHBs can be present simultaneously in both neoplastically transformed (hepatocellular carcinoma [HCC]) and nonneoplastic hepatocytes (eg, in idiopathic copper toxicosis).[13] So-called "hybrid" inclusions consist of both types,[4] suggesting similarities or even a direct relationship of their pathogeneses. It is believed that hybrid inclusions are formed whenever overexpression of

Abbreviations used in this paper: α1-AT, α_1-antitrypsin; ASH, alcoholic steatohepatitis; BSO, buthionine sulfoximine; DDC, 3,5-diethoxycarbonyl-1,4-dihydrocollidine; FFPE, formalin-fixed and paraffin-embedded; IF, intermediate filament; IHB, intracellular hyaline body; K8, keratin 8; K18, keratin 18; LCO, luminescent conjugated oligothiophene; LCP, luminescent conjugated polythiophene; MDB, Mallory–Denk body; NASH, nonalcoholic steatohepatitis; t-BHP, tertiary-butylhydroperoxide.

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p62 in combination with ubiquitin prevails over keratin. IHBs are probably generated by aggregation of overexpressed p62 and ubiquitin in the absence of abnormal keratins, whereas keratin and p62 are present at similar proportions in MDBs.[4,13] These findings suggest a yet unknown specific role for keratins in the formation of MDBs.

Using new tools to investigate differences in the molecular structure of the components present in the various types of inclusions can help in obtaining new insight into the nature and genesis of protein aggregates in liver diseases. Luminescent conjugated polythiophenes and oligothiophenes (LCPs and LCOs) have a swiveling thiophene backbone and were described as fluorescent ligands for amyloids. Their noncovalent binding to amyloid proteins constrains the rotational freedom of the flexible thiophene backbone and thereby alters the spectral properties of the LCP or LCO, depending on the conformation of the bound protein.[14,15] LCPs and LCOs were reported to be sensitive tools for detecting conformational variability in various amyloidogenic protein aggregates.[16–23] These luminescent ligands have also been used as conformation-sensitive optical probes for prion strain differentiation[17,19] and were recently applied for the characterization of amyloid-beta aggregates as well as neurofibrillary tangles, the 2 pathologic hallmarks of Alzheimer's disease.[16] Here we used LCOs to investigate changes in the molecular structure of MDB components in steatohepatitis and related in vivo and in vitro models and compared MDBs with other types of protein aggregates occurring in a variety of liver diseases.

Materials and Methods

If not stated otherwise, all chemicals were from Sigma-Aldrich (Vienna, Austria) or Merck (Vienna, Austria) and of analytical grade or better.

Cell Culture

CHO-K1 cells (CCL-61; American Type Culture Collection, Manassas, VA) were cultured in 75-cm^2 vented culture flasks using Ham's F-12 medium (PAA Laboratories, Pasching, Austria) supplemented with 10% fetal bovine serum, 4.4 g/L $NaHCO_3$, and 1% penicillin/streptomycin (PAA Laboratories). Before transfection, cells were cultured for at least 3 to 4 days.

Transfection

CHO-K1 cells were transfected with constructs for human *Krt8*, *Krt18*, and *p62* containing the cytomegalovirus enhancer promoter for constitutive expression.[4] Controls were transfected with empty pCDNA4/MycHis expression vector (Invitrogen, Groningen, The Netherlands). Transfection efficiency was determined in 3 independent experiments by counting green fluorescent protein–positive cells versus the total number of cells in 30 vision fields at 250× magnification. For details, see Supplementary Materials and Methods.

Induction of MDBs in Mice

Animal experiments were performed in accordance with national regulations under animal experimentation license. MDB formation was induced in male Swiss albino mice (strain Him-OF1; Institute of Laboratory Animal Research, Medical University of Vienna, Himberg, Austria) by feeding a diet containing 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC; 0.1% wt/wt) as described previously.[24] Three groups of mice (6 animals each) were kept on the DDC diet for 8 to 10 weeks, and 2 of these groups were left to recover from intoxication for 4 weeks, of which one group was reintoxicated with DDC for 3 days.[12,24] Heterozygous *krt8*$^{+/-}$ FVB/N mice were backcrossed for 10 generations with 129/Ola mice to obtain homozygous *krt8*$^{-/-}$ 129/Ola mice.[12,25] *krt18*$^{-/-}$ mice of 129/Ola genetic background were used.[26] MDBs were induced in 12-week-old *krt8*$^{-/-}$ and *krt18*$^{-/-}$ mice (both of 129/Ola background), weighing 25 to 30 g, by DDC feeding for 12 weeks.

Keratin Isolation and Circular Dichroism Spectroscopy

Keratin 8 (K8)/keratin 18 (K18) cytoskeleton from normal mouse liver homogenates was isolated using high ionic strength buffer and Triton X-100. Thereafter, K8 and K18 were separated by ion-exchange chromatography as described.[27] Circular dichroism spectra were collected separately for isolated K8 and K18 polypeptides. For details, see Supplementary Materials and Methods.

Immunocytochemistry of Transfected Cells

Transfected cells grown on glass coverslips were fixed in methanol and acetone (−20^{0}C), washed in phosphate-buffered saline (PBS), and incubated with mouse monoclonal anti-p62 antibody (BD Transduction Laboratories, Lexington, KY) or rat anti-K8 antibody (Troma I; Developmental Studies Hybridoma Bank, Department of Biology, University of Iowa, Iowa City, IA) diluted 1:100 in PBS. Coverslips were washed with PBS and incubated with secondary antibody (1:200, 1:300, Alexa 594; Molecular Probes, Leiden, The Netherlands) at room temperature for 30 minutes. h-HTAA and p-FTAA were diluted 1:500 in PBS, and coverslips were incubated for 20 minutes, washed with PBS, and mounted in mounting medium (Dako Cytomation, Glostrup, Denmark) containing 4′,6-diamidino-2-phenylindole.

Immunostaining of Tissues (Double- and Triple-Label Immunofluorescence)

Tissue cryosections (4 μm) were fixed with acetone or air dried without fixation (to exclude conformational changes induced by fixation) and stained with antibodies against p62, K8, or K18 followed by fluorescent-labeled secondary antibodies and LCOs. For details, see Supplementary Materials and Methods.

Immunostaining of Formalin-Fixed and Paraffin-Embedded Tissues (Double-Label Immunofluorescence)

Formalin-fixed and paraffin-embedded (FFPE) human liver sections were deparaffinized and washed with PBS for 10 minutes. h-HTAA (1:500 in PBS) was then applied to sections for 20 minutes. The sections were washed with PBS (3 times, 10 minutes) and microwaved in target retrieval solution, pH 9 (Dako), for 40 minutes at 150 W followed by blocking with 1% hydrogen peroxide in methanol. The sections were then immunostained using anti-K8 or anti-K18 antibodies (Leica Novocastra, Vienna, Austria) as primary and Alexa 594 anti-mouse secondary antibodies (Invitrogen, Eugene, OR).