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ORIGINAL PAPER

A cell culture system for the induction of Mallory bodies: Mallory bodies and aggresomes represent different types of inclusion bodies

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Abstract Mallory bodies (MBs) represent keratin-rich inclusion bodies observed in human alcoholic liver disease and in several chronic non-alcoholic liver diseases. The mechanism of their formation and their relationship to other inclusion bodies such as aggresomes is incompletely understood. We could induce keratin aggregates typical of MBs in cultured clone 9 rat hepatocytes by transgenic expression of wild-type and mutant aquaporin2 or α1-antitrypsin and under various forms of other cellular stress. By immunocytochemical analysis, p62 and poly-ubiquitin, components of classical MBs, could be demonstrated in the keratin aggregates of clone 9 hepatocytes. In addition, histone deacetylase 6, a microtubule-associated deacetylase, was identified as a novel component of the keratin aggregates. Thus, together with their ultrastructural appearance as randomly oriented, organelle-free aggregates of keratin filaments, the keratin aggregates in clone 9 hepatocytes correspond to MBs. An imbalance in keratin 8 to18 with very low levels of keratin 18 appears to be the underlying cause for their formation. The formation of MBs was microtubule-dependent although not depending on the activity of histone deacetylase 6. Forskolin-induced MBs in clone 9 hepatocytes were reversible structures which disappeared upon drug withdrawal. The MBs were not related to aggresomes since overexpressed misfolded transgenic proteins were undetectable in the keratin aggregates and no vimentin fiber cage was detectable, both of which represent hallmarks of aggresomes. Thus, cultured clone 9 hepatocytes are a useful system to study further aspects of the pathobiology of MBs.

Keywords Mallory bodies · Aggresomes · p62 · Keratin · Histone deacetylase 6 · Hepatocytes

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Introduction

Intermediate filament (IF) proteins such as keratins and vimentin are a major component of the cytoskeleton and play an important role in establishing the structure and integrity of the cell (Herrmann and Aebi 2004). They participate in many important cellular processes such as cell division, motility, cellular stress response, vesicular transport and protect cells against mechanical injury.

Keratins, the largest family of the IF cytoskeletal proteins, are divided into acidic keratin type I (K9–K20), and neutral to basic keratin type II (K1–K8) and have a unique tissue- and cell-specific expression (Moll et al. 2008). Epithelial cells express at least one type I, and one type II of keratin, forming non-covalently linked heteropolymers. Adult hepatocytes are unique since they express only keratin 8 and keratin 18 in a 1:1 stoichiometric ratio (Strnad et al. 2008).

A wide variety of human diseases are associated with alterations of the IF proteins (Omary et al. 2004). The formation of Mallory bodies (MBs) in hepatocytes is associated with alcoholic hepatitis and non-alcoholic steatohepatitis (Strnad et al. 2008). MBs consist of aggregates of hyperphosphorylated and/or poly-ubiquitinated

keratin 8 and 18 (Yuan et al. 1995). Moreover, non-keratin components such as hsp25, hsp70 and hsp90 (Zatloukal et al. 2002; Riley et al. 2003), as well as ubiquitin and p62 (Stumptner et al. 2002; Zatloukal et al. 2002) are constituents of MBs. In mice, MBs can be induced by chronic intoxication with griseofulvin (Denk et al. 1979), 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) (Yokoo et al. 1982), okadaic acid (Yuan et al. 1998) and dexamethasone (Yuan et al. 2000).

Recently, we reported (Hirano et al. 2002) that aggregates of keratin filaments exhibiting typical structural features of MBs existed in clone 9 rat hepatocytes stably expressing the ER-retained T126M-aquaporin2 (AQP2). Thus, we proposed that clone 9 rat hepatocyte cell cultures represent a first cell culture model for MB formation. The mechanism of formation of MBs in humans and mice as well as in the clone 9 hepatocytes is still incompletely understood. However, an imbalance in the relative amounts of keratin 8 and 18 as well as the keratin metabolism appears to be an important factor for MB formation in liver (Zatloukal et al. 2000; Nakamichi et al. 2005). It is also unknown if in the T126M-AQP2 expressing clone 9 hepatocytes, misfolded AQP2 is present in MBs.

Based on ultrastructural analyses, MBs in human alcoholic liver disease have been divided into three subtypes (Yokoo et al. 1972). MB subtype III was characterized by the absence of a well-defined filamentous structure and the presence of highly electron dense granular or homogeneous inclusions, which resemble aggresomes (Johnston et al. 1998; Garcia-Mata et al. 1999; Kopito and Sitia 2000). Aggresomes are cytoplasmic inclusion bodies observed in several non-hepatic diseases such as in some myopathies (Goebel 2003), in Alexander disease (Hagemann et al. 2006), and in neurodegenerative diseases including Alzheimer and Parkinson disease as well as in amyotrophic lateral sclerosis (Cairns et al. 2004). They are formed when the proteolytic capacity of the proteasome is exhausted and misfolded proteins aggregate in the cytosol (Johnston et al. 1998; Garcia-Mata et al. 1999; Kopito and Sitia 2000). They are composed of poly-ubiquitinated misfolded proteins that are surrounded by a cage of vimentin fibers (Johnston et al. 1998). Recently, histone deacetylase 6 (HDAC6), a microtubule-associated deacetylase, was identified as a component of aggresomes. It acts to recruit misfolded proteins to dynein motors for transport to aggresomes (Kawaguchi et al. 2003). Thus, this enzyme plays an important role in the intracellular transport of misfolded proteins.

In the present paper, we studied aspects of the mechanism of MB formation in cultures of clone 9 hepatocytes, and analyzed their composition in regard to the presence of non-keratin components and established their relationship to aggresomes.

Materials and methods

Antibodies and reagents

A guinea pig anti-p62 antibody (Zatloukal et al. 2002) was kindly provided by Dr. Helmut Denk (Medical University of Graz, Austria) and a rabbit anti-human HDAC6 antibody (Hubbert et al. 2002) by Drs. Y. Kawaguchi and P. Y. Tao (Duke University NJ, USA). Mouse monoclonal anti-human Lu-5 Pan-Vertebrate conserved cytokeratin, and Scriptaid were obtained from Biochemical (Augst, Switzerland), mouse monoclonal anti-keratin 8 and 18 antibodies from Progene Biotechnik (Heidelberg, Germany), mouse monoclonal anti-vimentin antibody from Neomarkers (Fremont, CA, USA), mouse monoclonal anti-γ tubulin and monoclonal anti-β tublin from Sigma (Buchs, Switzerland) and mouse monoclonal anti-multi-ubiquitin antibody from MBL Co. (Nagoya, Japan). Affinity-purified rabbit polyclonal anti-rat aquaporin2 (AQP2) antibody was raised against a synthetic peptide comprising the carboxy terminal residues 254-271 of rat AQP2 cross-reactive with human AQP2. Polyclonal anti-human α1-antitrypsin antibody was purchased from DAKO (Zug, Switzerland), Alexa 488-conjugated $(Fab)_2$ fragments of goat anti-mouse IgG and Alexa 546 conjugated $(Fab)_2$ fragments of goat anti-rabbit IgG from Molecular Probes (Eugene, OR, USA), and alkaline phosphatase-conjugated goat anti-mouse IgG from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA).

TRI reagent was purchased from Lucerna Chem (Lucerne, Switzerland), reverse transcription system from Promega (Wallisellen, Switzerland), *Taq* DNA polymerase, lipofectamine 2000, cell culture media and fetal bovine serum from Invitrogen (Basel, Switzerland), and QIAquick PCR-purification Kit from QIAGEN (Basel, Switzerland). Restriction enzymes, T4 DNA ligase and protease inhibitor tablets were from Roche Diagnostics (Rotkreuz, Switzerland). Oligonucleotides were synthesized by Microsynth (Balgach, Switzerland). Silencer siRNA construction kit was from Ambion Inc. (Applied Biosystems, Rotkreuz, Switzerland). Lactacystin, forskolin, griseofulvin, nocodazole and trichostatin A were obtained from Sigma (Buchs, Switzerland), ALLN and MG132 from Calbiochem (Lucerne, Switzerland).

Cell lines and transfections

Cell lines used (Table 1) were obtained from American Type Culture Collection (Manassas, VA) and grown in recommended medium containing 10% fetal bovine serum at 37°C in a 5 and 10% CO_2 incubator, respectively. Clone 9 hepatocytes represent a heterogeneous cell population regarding expression of keratin. Therefore, we established

Table 1 Characterization of IF cytoskeletal proteins in various cell lines

Cell line	Cytokeratin[a]	Vimentin	MB[b]	Aggresomes[b]
HEK 293	+	+	–	
HeLa	+	+	–	
HT29	+	+	–	
CaCo-2	+	+		
CHO-K1	–	+	–	+[d]
Clone 9 hepatocytes #16	+	+	+	
Clone 9 hepatocytes #14	+	+	+	
Clone 9 hepatocytes #11	–	+	–	
Clone 9#16 expressing wt-AQP2	+	+	+	–[c]
Clone 9#16 expressing T126M-AQP2	+	+	+	–[c]
Clone 9#16 expressing A1AT (HK)	+	+	+	–[c]
CHO-K1 expressing T126M-AQP2	–	+	–	–[c]
CHO-K1 expressing A1AT (HK)	–	+	–	–[c]

[a] Cytokeratin was detected with the monoclonal anti-human Pan cytokeratin antibody Lu-5

[b] MB were induced by treatment of the cells with the proteasome inhibitor MG132 (10 μM) for 3–24 h

[c] Presence of aggresomes was analyzed by imunofluorescence for AQP2 or α1-antitrypsin

[d] Aggresomes in non-transfected CHO cells were formed following incubation with MG132 (40 μM) for 3 h

clonal keratin-positive cell lines by limiting dilution. These clonal lines of clone 9 hepatocytes were used in all our studies. Transfections with different aquaporin2 (AQP2)- and α1-antitrypsin-constructs were performed as described previously (Hirano et al. 2002; Torossi et al. 2006). Clonal cell lines were generated in 96-multiwell plates and individual clones were tested for expression of AQP2 and α1-antitrypsin by immunofluorescence.

Treatments of clone 9 rat hepatocytes

Clone 9 hepatocytes were incubated in medium containing 1, 2 and 5% ethanol, respectively, for 2 h, or with griseofulvin (10 nM) for 1–2 days, or with forskolin (10 μM) for 6 h at 37°C. Clone 9 hepatocytes stably expressing wild-type AQP2, T126M-AQP2 or the Hong Kong variant of α1-antitrypsin were treated with the proteasome inhibitors MG132 (10 μM), lactacystin (50 μM) or ALLN (100 μM) for 3 h up to 24 h at 37°C. Clone 9 hepatocytes were treated with MG132 (10 μM) for 3 h and additionally with nocodazole (10 μM) or taxol (10 μM) for 1, 2 and 3 h to depolymerize microtubules. To inhibit histone deacetylase 6, cells were pretreated with MG132 (see above) followed by tricostatin A (1 or 10 μM) or scriptaid (1 or 10 μM) for 16 h at 37°C.

Confocal immunofluorescence microscopy

Clone 9 rat hepatocytes grown on glass cover slips were formaldehyde-fixed and saponin-permeabilized as described previously (Hirano et al. 2002). Single immunofluorescence for keratin using the pan cytokeratin Lu-5 monoclonal antibody and for vimentin was performed (Hirano et al. 2002). Double immunofluorescence for keratin in combination with either p62, poly-ubiquitin, vimentin, AQP2, α1-antitrypsin or HDAC6 was performed according standard protocol using appropriate fluorescent secondary antibodies. Immunofluorescence was observed and recorded with a Leica Confocal Laser Scanning Microscope TSC PS2 using the 100× objective (1.4). The *z*-axis resolution of this equipment was at maximum 300 nm per voxel and the *x*; *y* settings were between 50 and 250 nm per voxel.

Electron microscopy and immunoelectron microscopy

For standard transmission electron microscopy, cells were double aldehyde-fixed followed by osmium tetroxide fixation and Epon embedding (Hirano et al. 2002). For immunogold labeling, cells were processed as described previously (Hirano et al. 2002). Briefly, they were fixed in situ 3% paraformaldehyde in PBS, mechanically removed with a rubber policeman and resuspended in PBS containing 0.5% BSA. After centrifugation, cells were enclosed in 2% low-melting agarose and infiltrated with sucrose (2.0 M) containing 15% polyvinol pyrrolidone (10 kDa) in the presence of 1% paraformaldehyde. Ultrathin cryosections were prepared according to Tokuyasu (Tokuyasu 1978, 1980). For immunogold labeling, grids with attached ultrathin cryosection were washed with PBS and conditioned with PBS containing 1% BSA and 0.01% Tween 20. Afterwards, grids were incubated either with anti-keratin 8 or anti-HDAC6 antibodies for 2 h at ambient temperature, rinsed with PBS, and incubated with 8 nm gold-labeled

goat anti-mouse IgG for 1 h. For double-labeling experiments, cryosections were simultaneously incubated with mouse anti-keratin 8 and guinea pig anti-p62 antibodies, followed by 12 and 8 nm gold-labeled appropriate secondary antibodies, respectively.

RT-PCR

For the detection of keratin 8/18 mRNA and rat HDAC6 mRNA, RT-PCR was performed. Total RNA isolated from rat liver or from clone 9 hepatocytes was reverse transcribed by AMV reverse transcriptase using random primers. In the subsequent PCR amplification, the following specific oligonucleotides were used: kertin 8; forward 5′-CAG AAA GCC AAG CAG GAC AT-3′; reverse 5′-CAA TCT TCC CGA CCA CA-3′, K18; forward 5′-TTG CCG CTG ATG ACT TTA GA-3′; reverse 5′-TCC AGT TCC TCA CGG TTC TT-3′, rat HDAC6; forward 5′-CGG CCA AGA TTC TTC TAC CAG G-3′; reverse 5′-CCG AAT CTC AGC CCC CAT AAC T-3′. As control, rat β-actin was amplified using the following primers: forward 5′-CTG ATC CAC ATC TGC TGG AAG GTG G-3′; reverse 5′-ACC TTC AAC ACC CCA GCC ATG TAC G-3′. The PCR products were resolved in 1% agarose gel containing ethidium bromide.

Isolation of keratin filaments and Western blotting

Keratin filaments were isolated according to the protocol of Gilmartin et al. (1984). Briefly, clone 9 hepatocytes were lysed in 1% Triton X-100, 25 mM Tris–HCl buffer (pH 7.4) containing protease inhibitors. The buffer-insoluble pellet was washed in the same buffer followed by incubation in 1 M KCl, 25 mM Tris–HCl buffer (pH 7.4) at 37°C for 30 min. The insoluble pellet obtained after centrifugation at 12,000×g for 5 min was extracted in 9 M urea, 100 mM β-mercaptoethanol, 25 mM Tris–HCl buffer (pH 7.4) containing protease inhibitors at 37°C for 30 min. Soluble proteins (100 µg protein/lane) were separated in 12% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes using a semi-dry blotting apparatus (Towbin et al. 1979). Western blot analysis was done with anti-keratin 8 (diluted to 1 µg/ml) and anti-keratin 18 (diluted to 1 µg/ml) antibodies, respectively, followed by alkaline phosphatase-conjugated secondary antibodies. Color reaction was performed using nitro blue tetrazolium/BCIP as substrates.

Inhibition of HDAC6 by RNA-mediated interference

For RNA-mediated interference, the following siRNA oligonucleotides of rat HDAC6 were designed according to the program provided by Ambion Inc. (Applied Biosystems, Rotkreuz, Switzerland); forward: 5′-AAA GAA TGA GTC ACT GCA CCT GTC TC-3′; reverse 5′-AAT GCA GTG ACT CAT CAT TCT CCT GTC TC-3′; forward 5′-AAA GAC AGC TAA GGC ATT GCT CCT GTC TC-3′; reverse 5′-AAA GCA ATG CCT TAG CTG TCT CCT GTC TC-3′. Clone 9 hepatocytes were plated on 35-mm^2 Petri dishes to about 70% confluency and transiently transfected with 10 nM of siRNA using 3-µl Lipofectamine 2000 reagent. The effect of RNA interference for HDAC6 was determined by immunofluorescence and by RT-PCR.

Results

Mallory body formation is not a general feature of keratin-positive cell lines

Recently, we reported an in vitro model for MB formation using cultures of clone 9 rat hepatocytes stably expressing ER-retained mutant T126M-aquaporin 2 (AQP2) (Hirano et al. 2002). Additional proteasome inhibition with MG132 resulted in an increase in the number of keratin aggregates in transfected clone 9 hepatocytes as well as in non-transfected cells (Hirano et al. 2002). A detailed analysis by conventional transmission electron microscopy of the keratin immunoreactive aggregates (Hirano et al. 2002) revealed fine structural features typically observed in MB in human alcoholic liver disease and griseofulvin-intoxicated mice (Strnad et al. 2008). In Fig. 1, a clone 9 hepatocyte with a large perinuclear aggregate consisting of a meshwork of loosely arranged, randomly oriented filaments is observed. This aggregate of filaments is free of cellular organelles (Fig. 1) and immunoreactive for keratin (see below). Based on our previous findings, we asked whether such keratin aggregates can be induced in other epithelial cell lines. For this purpose, we first investigated the keratin expression in HEK 293 cells, HeLa cells, human colon carcinoma cells HT29 and Caco-2 cells and in CHO-K1 cells (Table 1). We next tested whether aggregation of keratin can be observed in the other keratin-positive cell lines. Incubation of HeLa, HEK 293 and HT29 cells with the proteasome inhibitor MG132 for 3–48 h resulted in a redistribution of keratin filaments. However, no MB-like aggregates of keratin filaments were detected (data not shown). Thus, MB formation is not a general feature of cells containing keratin filaments.

Keratin 18 is undedectable in clone 9 hepatocytes

To verify whether an imbalance in keratin 8 to 18 levels is underlying the formation of keratin aggregates in the cloned lines of clone 9 hepatocytes, the keratin 8 and 18 mRNA levels were determined in individual clones.

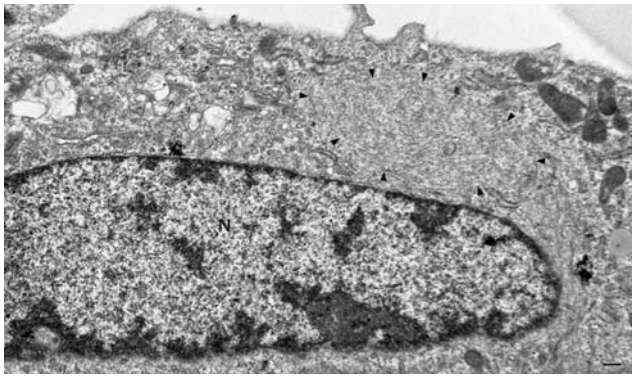


Fig. 1 A clone 9 hepatocyte containing a large perinuclear aggregate of filaments (marked by *arrowheads*). Cellular organelles are excluded. *N* nucleus, *bar* 0.5 µm

Undoubtedly, the mRNA level of keratin 18 was either drastically lower than that of keratin 8 or undetectable, in contrast to the reported 1:1 ratio for adult rat liver (Ku et al. 1999; Omary et al. 2002) (Fig. 2a). Consistently, only keratin 8 but not keratin 18 was detected by Western blot in all studied clonal lines of clone 9 hepatocytes (Fig. 2b). Likewise, keratin 8 immunolabeling was obtained by immunofluorescence (data not shown) and by immunoelectron microscopy in keratin bundles (Fig. 2c) and in the large aggregates of filaments of clone 9 hepatocytes (Fig. 2d). In adult rat liver and in HeLa cells, both serving as a positive control, keratin 8 and 18 were detected by Western blot (Fig. 2b). Thus, the low expression of keratin 18 in clone 9 hepatocytes might be an important factor for the formation of aggregates of keratin filaments corresponding to MBs.

Cellular stress induces Mallory bodies in clone 9 rat hepatocytes

MBs are observed in human chronic alcoholic liver diseases and can be induced in mice under conditions of chronic intoxication (Denk et al. 1979, 2000; Omary et al. 2002; Zatloukal et al. 2004) and in clone 9 hepatocytes by expressing an ER-retained misfolded membrane protein, T126M variant of AQP2 (Hirano et al. 2002; present in Fig. 3a–c). Furthermore, we found that overexpression of the ER-retained Hong Kong variant of α1-antitrypsin in clone 9 hepatocytes (Torossi et al. 2006) induced MBs (Fig. 3d, f). Hence, a misfolded membrane as well as a misfolded secretory protein can induce MB in clone 9 hepatocytes. We have tested other forms of cellular stress for their capacity to induce MBs in clone 9 hepatocytes. After short time incubation with 1 and 2% ethanol, respectively, a two to fourfold increase of cells containing keratin aggregates was observed (Fig. 4a). Trypan blue staining showed that such a treatment had no effect on cell viability. On the other hand, treatment of clone 9 hepatocytes with griseofulvin, used to induce MBs in mice liver (Denk et al. 1979), did not result in the formation of keratin aggregates in clone 9

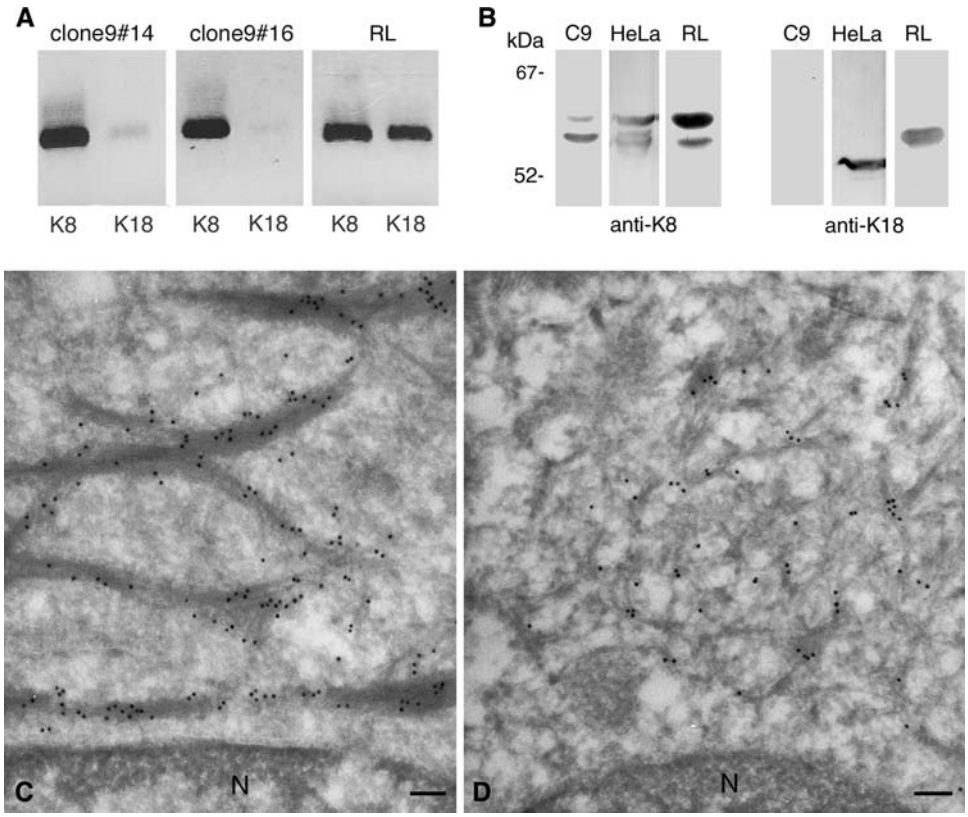


Fig. 2 Keratin 18 is undetectable in clone 9 hepatocytes. **a** RT-PCR amplification was performed using RNA isolated from clonal keratin-positive clone 9 hepatocytes and rat liver and specific keratin 8 and keratin 18 primers (see "Materials and methods"). **b** Western blot analysis of isolated keratin filaments from clonal keratin-positive clone 9 hepatocytes (C9), HeLa cells and rat liver (RL) using anti-keratin 8 and anti-keratin18 antibodies. **c**, **d** Immunoelectron microscopic detection of keratin 8 in keratin bundles (**c**) and in a MB (**d**) of clone 9 hepatocytes expressing T126M-AQP2. *N* nucleus, *bars* 200 nm (**c**, **d**)

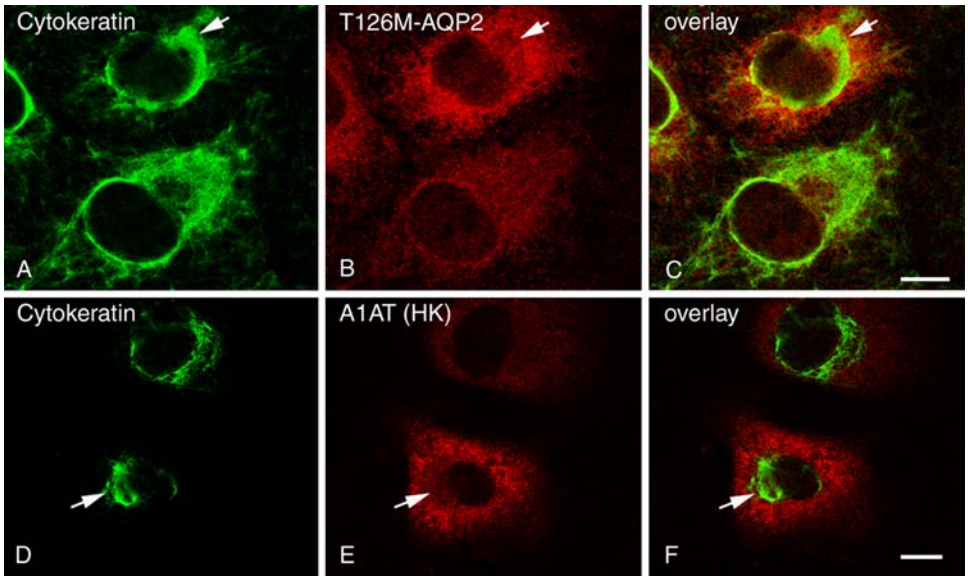


Fig. 3 Mallory bodies are induced but do not contain mutant AQP2 or Hong Kong variant of α1-antitrypsin. **a–c** Double confocal immunofluorescence labeling for keratin and AQP2 shows that MBs in clone 9 hepatocytes expressing T126M-AQP2 and treated with MG132 do not contain mutant AQP2. **d–f** Likewise, MBs in clone 9 hepatocytes overexpressing the Hong Kong variant of α1-antitrypsin do not contain mutant α1-antitrypsin. (*bars* 10 μm)

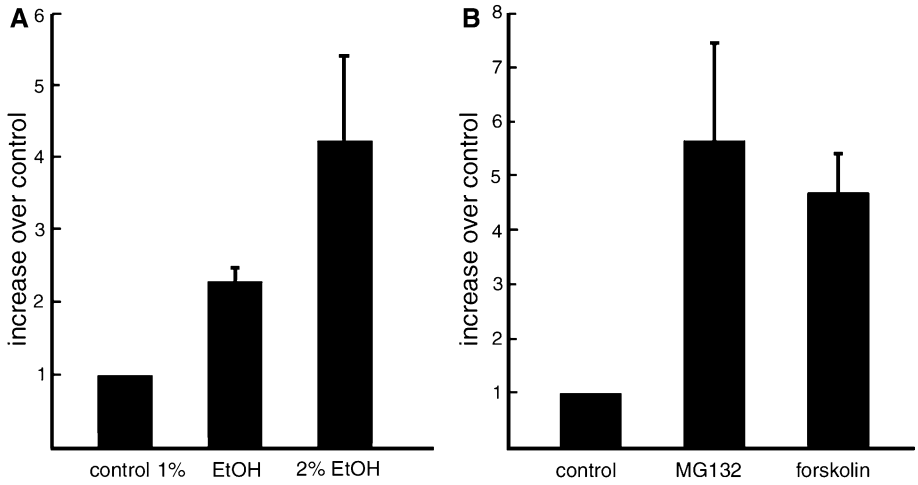


Fig. 4 Cellular stress induces Mallory bodies in clone 9 hepatocytes. **a** Mallory bodies can be induced by culturing cells in the presence of ethanol for 2 h. **b** Treatment with the proteasomal inhibitor MG132 (10 μM) or with forskolin (10 μM) for 3 h results in the formation of Mallory bodies. Mallory body formation was evaluated by immunofluorescence for keratin (see "Materials and methods") (mean ± SD are based on at least five independent sets of experiments)

hepatocytes. Furthermore, the number of clone 9 hepatocytes containing keratin aggregates increased approximately sixfold upon treatment with the proteasome inhibitors MG132 (Fig. 4b), lactacystin or the tetrapeptide ALLN (data not shown), indicating that MBs can be induced when the proteasomal degradation of cellular proteins is inhibited.

Mallory body formation in clone 9 hepatocytes is reversible

MBs are characterized by the accumulation of hyper-phosphorylated keratin filaments (Stumptner et al. 2000). Forskolin is known to increase intracellular cAMP levels and to trigger protein kinase (PKA) activities and has been shown to facilitate the hyperphosphorylation of keratin filaments in colon carcinoma Caco-2 cells (Baricault et al. 1994). In clone 9 hepatocytes, the number of cells containing keratin aggregates was increased about fivefold after forskolin treatment (Fig. 4b). After removal of forskolin, a time-dependent decrease in the number of keratin aggregate-containing cells was observed (Fig. 5). Three hours following forskolin removal, a redistribution of keratin filaments occurred and a keratin network undistinguishable from that of non-treated cells developed. We could exclude that the decrease in the number of MBs was due to a mitotic increase of the cell number. Measuring the cell growth kinetics gave similar values in the absence or presence of forskolin (Fig. 5, inset) indicating that the decrease in the number of MBs was significant.

Mallory bodies in clone 9 hepatocytes contain p62 and poly-ubiquitin

p62 and poly-ubiquitin are major non-keratin constituents of MBs of chronic alcoholic liver disease in humans (Zatloukal et al. 2002). We investigated whether they were also present in the induced keratin aggregates of clone 9 hepatocytes. By double immunofluorescence, p62 was

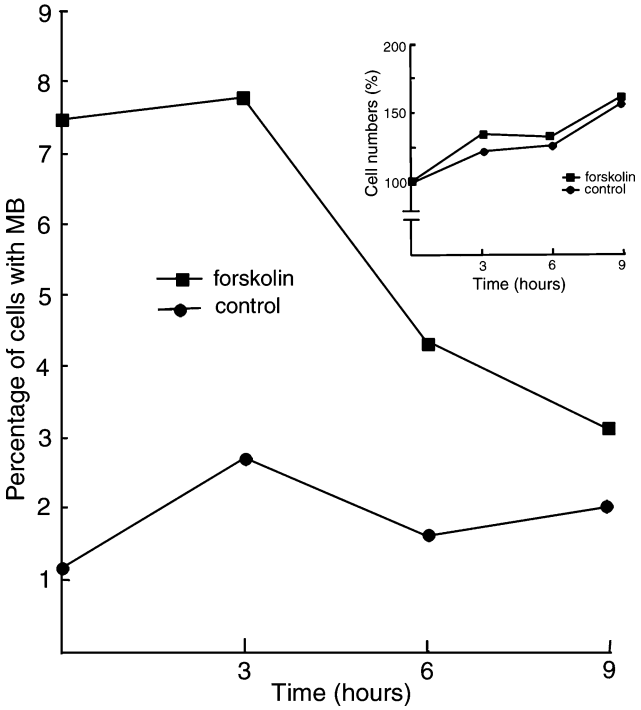


Fig. 5 Mallory bodies induced by forskolin are reversible. Clone 9 hepatocytes were incubated for 3 h in medium containing forskolin (10 μM) to induce MBs (time point 0). Afterwards, the cells were washed and maintained in medium not supplemented with forskolin for various time periods. The growth kinetics of control and forskolin-treated cells were similar (*inset*). (At least three independent sets of experiments were performed)

detected in the keratin aggregates (Fig. 6a–c). These results could be confirmed by double immunogold labeling (Fig. 6d, e). Furthermore, the keratin aggregates exhibited immunoreactivity for poly-ubiquitin (Fig. 6f, h). On the other hand, the IF-type vimentin (Table 1), regularly associated with aggresomes, was not detected as a component of the keratin aggregates of clone 9 hepatocytes (Fig. 6i–l).

Misfolded membrane and secretory proteins are not detectable in Mallory bodies

Wild-type and T126M-AQP2 were stably expressed in keratin-positive clone 9 hepatocytes (Table 1) in the presence of the proteasome inhibitor MG132. Both wild-type and mutant AQP2 were undetectable in MB by double immunofluorescence (Fig. 3a–c). Likewise, MBs induced by expressing the Hong Kong variant of α1-antitrypsin, in the presence of MG132 did not contain the misfolded protein (Fig. 3d–f). These data demonstrate that although MBs in clone 9 hepatocytes can be induced by the expression of misfolded proteins, the aberrant transgenic proteins are not contained in the MBs. Remarkably, under our experimental conditions no aggresomes were formed

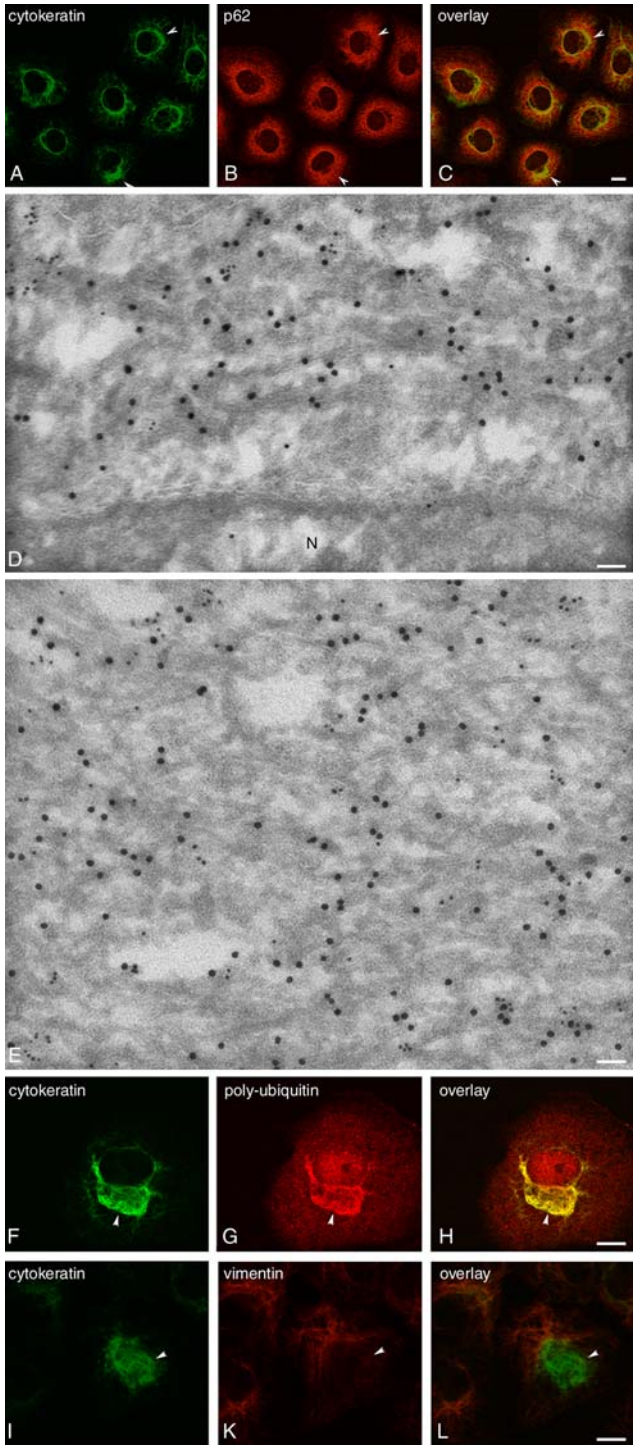


Fig. 6 Mallory bodies contain p62 and are positive for poly-ubiquitin. **a–c** Double confocal immunofluorescence microscopy demonstrates presence of p62 in MBs of clone 9 hepatocytes expressing T126M-AQP2. **d**, **e** Immunogold double labeling for keratin 8 (large gold particles) and p62 (small gold particles) in MBs of clone 9 hepatocytes expressing T126M-AQP2. **f–h** MBs in clone 9 hepatocytes are positive for poly-ubiquitin. **i–l** Immunofluorescence for vimentin indicates exclusion of vimentin from the keratin positive MBs. *Arrowhead* indicates a MB. *Bars* 10 μm (**c**, **h**, **l**), 50 nm (**d**, **e**)

in clone 9 hepatocytes or in CHO-K1 cells expressing mutant AQP2 or the Hong Kong variant of α1-antitrypsin, respectively (Table 1).

Histone deacetylase 6 is a component of Mallory bodies

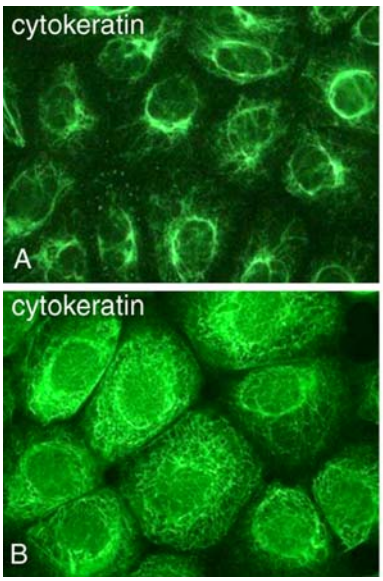
Keratin and γ-tubulin are co-localized in clone 9 hepatocytes, indicating that the aggregation of keratin filaments occurred at the microtubule organizing center (Schatten 2008). Treatment of clone 9 hepatocytes with the microtubule-disrupting agents, nocodazole or taxol, changed the distribution of keratin filaments dramatically. Furthermore, the number of MBs in cells treated either with nocodazole or taxol and in addition with MG132 was reduced as compared to only MG132-treated cells (Fig. 7). These data indicated that microtubule disrupting reagents prevent MB formation. Recently, HDAC6 was identified as a component of aggresomes. It acts to recruit misfolded protein cargo to dynein motors for subsequent transport to aggresomes (Kawaguchi et al. 2003). To investigate whether HDAC6 is a component of MBs in clone 9 hepatocytes, double immunolabeling for HDAC6 and keratin was performed. HDAC6 and keratin were co-localized in MBs of clone 9 hepatocytes (Fig. 8a–c). By immunoelectron microscopy, HDAC6 was also detectable in MBs (Fig. 8d).

Thus, we hypothesized that HDAC6 might act in the recruitment of aggregated keratin filaments for MBs. However, inhibition of HDAC6 using trichostain A or scriptaid (Su et al. 2000; Hubbert et al. 2002) did not reduce the number of MBs in clone 9 hepatocytes (data not shown). Similarly, the knock-down of rat HDAC6 with different siRNA constructs had no effect on MB formation (Fig. 8e, f). Thus, we concluded that HDAC6 is probably not involved in the recruitment of keratin filaments in MBs.

Discussion

Mallory bodies are typically observed in humans with alcoholic and non-alcoholic liver disease and are also present in the hepatocytes of patients with steatohepatitis as well as in hepatocellular carcinomas (Okamura 1976; Denk et al. 1979; French 1983), in experimental mice models (Denk et al. 1979; Franke et al. 1979; Denk and Franke 1981; Yuan et al. 1998; Yuan et al. 2000) and in clone 9 hepatocytes expressing an ER-retained misfolded polytope membrane protein (Hirano et al. 2002). Although MBs are important in the diagnosis of several liver disorders, the mechanism of their formation is not fully understood. In the present study, we used the clone 9 hepatocytes as a model (Hirano et al. 2002) for MB formation in order to study conditions leading to their formation, their turnover and composition as well as their relationship with aggresomes.

We found that MB can be induced in clone 9 hepatocytes by various types of cellular stress such as proteasome inhibition, overexpression of a misfolded secretory and a membrane protein, and by treatment with various chemical compounds. Under basal conditions, cells refold misfolded proteins assisted by chaperones, or degrade them via the ubiquitin-proteasome system (Brodsky and McCracken 1999). Our data demonstrated that the presence of misfolded proteins or experimental proteasome inhibition causes formation of intracellular inclusion bodies typical of MBs in clone 9 hepatocytes. Similarly, during in vivo proteasome inhibition, an accumulation of keratin, polyubiquitinated proteins and heat shock proteins was observed in rats (Bardag-Gorce et al. 2004). Ethanol treatment of clone 9 hepatocytes induced MBs efficiently, which might be either due to the decreased proteasome activity or by oxidative stress caused by ethanol-metabolism species. Ethanol



C

	Number of MBs (%)
MG132, 3h	100
MG132, 3h + nocodazole 1h	95.2
MG132, 3h + nocodazole 2h	43.8
MG132, 3h + nocodazole 3h	20.4
Non-treated	22.1

Fig. 7 Nocodazole treatment prevents Mallory body formation. **a** MBs in clone 9 hepatocytes, induced after treatment with MG132. **b** Although MG132 and nocodazole treatment dramatically changed the distribution of keratin filaments, MBs were not observed. **c** Time-dependent decrease of MBs in clone 9 hepatocytes after nocodazole and MG132 treatment, reaching the number of non-treated cells. Number of MBs induced after MG132 treatment was set as 100%

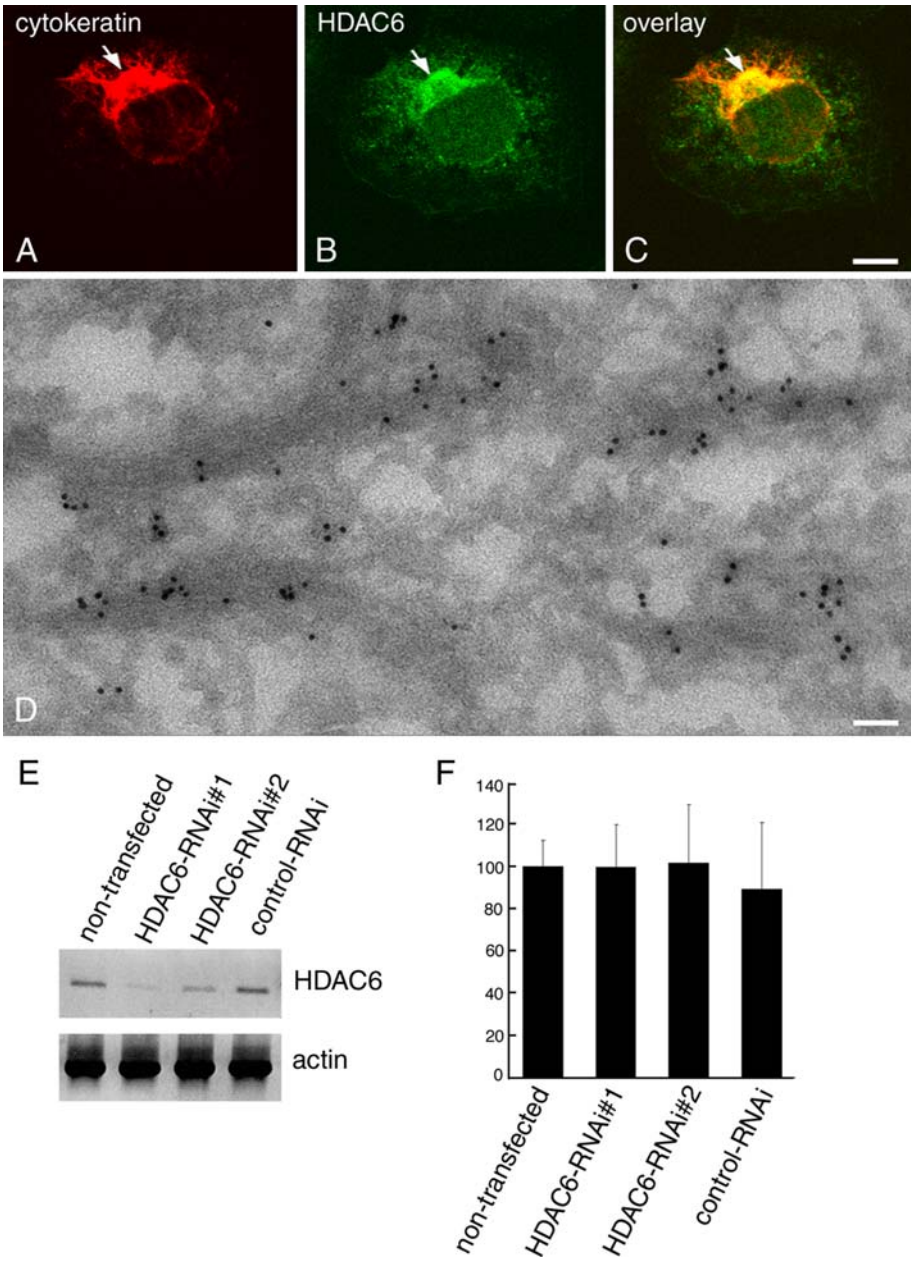


Fig. 8 Histone deacetylase 6 (HDAC6) is a component of Mallory bodies. **a**–**c** Double confocal immunofluorescence showed co-localization of keratin and HDAC6 in clone 9 hepatocytes expressing T126M-AQP2. **d** Immunogold labeling for HDAC6 in MBs. Ultrathin frozen section. **e** Knock down of HDAC6 in clone 9 hepatocytes by transfection with RNAi-constructs. RT-PCR analysis showed an almost complete absence of HDAC6 mRNA (*lanes* 2 and 3). No change in HDAC6 expression was observed in non-transfected clone 9 hepatocytes (*lane* 1) or after transfection with control RNAi (*lane* 4). Rat β-actin was used as control for RT-PCR. **f** Quantification of the HDAC6 knock down on the number of MBs in clone 9 hepatocytes induced by MG132. siRNA knock down had no effect on the number of MBs (*lanes* 2, 3), alike the use of control RNAi (*lane* 4) (mean ± SD are based of at least five independent sets of experiments). *Bars* 10 µm (**c**), 100 nm (**d**)

treatment of transformed HepG2 cells for 24 h has been shown to reduce the gene expression of the 26 S proteasome subunit and to induce keratin 8 and 18 expression (Bardag-Gorce et al. 2006). Forskolin which causes hyperphosphorylation of keratin filaments (Baricault et al. 1994) induced MBs in clone 9 hepatocytes. It allowed us to demonstrate that MB formation is a reversible process in clone 9 hepatocytes. It was not unexpected that griseofulvin did not induce inclusion bodies of the MB type in our cell culture system since chronic intoxication of mice is a required condition (Denk et al. 1979). Our data unequivocally demonstrated that keratin 8 is the major component of the inclusion bodies of clone 9 hepatocytes. They are composed of a dense network of randomly oriented keratin filaments and devoid of cytoplasmic organelles (Hirano et al. 2002), which is the canonical fine structural appearance of MBs in alcoholic liver disease (Strnad et al. 2008). By reverse transcription polymerase chain reaction analyses a vast excess of keratin 8 mRNA over keratin 18 mRNA in clone 9 hepatocytes was revealed and this was confirmed at the protein level by Western blot. However, despite levels of keratin 18 often too low to be detectable by Western blot, a keratin network is observed in clone 9 hepatocytes. On the other hand, the low amount of K18 protein seems to be an important pathogenic factor for MB formation in clone 9 hepatocytes. An imbalance of keratin 8 and 18 has been shown to dysregulate the keratin metabolism (Kulesh and Oshima 1988; Kulesh et al. 1989) and to cause diseased

states (Baribault et al. 1993; Baribault et al. 1994). Moreover, aged keratin 18 knock-out mice developed a distinct liver pathology with keratin 8-positive MB-like aggregates (Magin et al. 1998), while in keratin 8-null mice, no MBs were detectable after chemical intoxication (Zatloukal et al. 2000). The importance of the keratin 8/18 stoichiometry was also demonstrated using cultured hepatocytes. Overexpression of wild type or a mutant form of keratin18 resulted in MB formation because of the tendency of keratin 18 to aggregate, whereas co-expression of keratin 8 suppressed this tendency (Nakamichi et al. 2002; Kumemura et al. 2004). Hence, dysregulated expression of keratin 18 or an imbalance between keratin 8 and 18 seems to be a central condition for MB formation.

The keratins 8 and18 in MBs of human alcoholic hepatitis and in mouse models carry several posttranslational modifications including phosphorylation, acetylation, cross-linking and ubiquitination (Denk et al. 1979, 2000; Ohta et al. 1988; Zatloukal et al. 1992; Ku and Omary 2000). Phosphorylation appears to be an important modification of keratins since it is involved in keratin filament organization and interaction with binding proteins (Ku et al. 1999; Omary et al. 2006). Consistent with these in vivo observations, keratins in MBs of clone 9 hepatocytes appear to be hyperphosphorylated and are poly-ubiquitinated. Furthermore, we identified p62 (Zatloukal et al. 2002) in MBs of clone 9 hepatocytes. p62 can bind poly-ubiquitinated proteins and targets them for degradation by proteosomes or autophagy (Seibenhener et al. 2004; Bjorkoy et al. 2005) and is involved in MB formation (Nan et al. 2004). In conclusion, the MBs formed in the clone 9 hepatocyte cell culture system appear to be compositional identical to those found in human alcoholic liver disease and in animals induced by chronic intoxication.

MBs and aggresomes are cytoplasmic inclusion bodies found under various diseased states. They are composed of poly-ubiquinated insoluble aggregates of proteins, which have escaped proteasomal degradation. Although it was suggested that MBs found in chronic liver disease are aggresomes (French et al. 2001; Bardag-Gorce et al. 2002; Riley et al. 2002), we believe that they have to be distinguished from aggresomes. In contrast to aggresomes which are composed of misfolded aggregated proteins surrounded by a vimentin cage (Johnston et al. 1998), MB in clone 9 hepatocytes expressing misfolded AQP2 or α1-antitrypsin was not only devoid of the overexpressed transgenic proteins but also had no cage-like vimentin structure. From this we conclude that MBs and aggresomes are different types of inclusion bodies. Despite, there are some common features. Microtubles are involved in the formation of both aggresomes (Johnston et al. 1998; Cardinale et al. 2001; Kabore et al. 2001) and of MBs (present study). HDAC 6 is a component of both types of inclusion bodies (Kawaguchi et al. 2003; present study) but seems to be involved only in the formation of aggresomes.

In summary, the keratin aggregates formed in clone 9 hepatocytes correspond to MBs based on their fine structure and protein composition. They can be induced by various forms of cellular stress and are reversible. The present study also demonstrated that MBs are inclusion bodies different from aggresomes.

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