Does Aging Affect Liver Microtubules? (43378)

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Abstract. **Microtubules are essential for many cell processes, e.g., ligand-receptor endocytosis and the vectorial movement of endosomes. The cytoskeleton, particularly microtubules, may undergo age-related changes that are reflected in cell dysfunctions. For example, the translocation of ^{125}I-IgA-containing vesicles from the sinusoidal surface to the pericanalicular cytoplasm is reduced (>40%) in old versus young rats. Electron microscopic analysis demonstrated that the concentration of microtubule profiles in young animals is within 10–20% of that in old rats. The relative concentration of polymerized tubulin declines >70% by 12 months of age, but the total tubulin content remains unchanged until later, i.e., declining 50% by 24 months. Concomitant increases occur in the free fractions of microtubule-associated proteins (MAP), i.e., MAP$_1$ and heat-stable MAPS. These fractions are not associated with polymerized tubulin. The declines in total and polymerized tubulin, together with the increases in the MAPS' free fractions, may be indicative of fewer and/or shorter microtubules. These data lend credence to the supposition that aging is accompanied by perturbations of microtubule functions that ultimately are expressed as biomarkers characteristic of aging.**

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Cytoplasmic microtubules are essential for a variety of cellular processes, including ligand-receptor endocytosis and the subsequent vectorial movement of ligand-containing vesicles destined for secretion or degradation (see Ref. 1 for a review). The maintenance of these functions depends on a stable polymer to monomer tubulin ratio, an autoregulated process (2–4). Caron *et al.* (5) have shown that the autoregulation of tubulin synthesis and the processing of endocytosed ligands are sensitive to small changes in this critical ratio. Since tubulin constitutes approximately 1% of the total hepatic protein composition and aging is accompanied by shifts in the synthesis and intracellular concentrations of numerous hepatic proteins, age-associated changes in the rate of tubulin synthesis or in the tubulin polymer to monomer ratio may contribute to concomitant alterations in microtubule-dependent functions (6; see Ref. 7 for a review).

There is some evidence that the cytoskeletal system, particularly microtubules, undergoes age-related changes that are reflected in cellular dysfunctions (8). In rat hepatocytes, for example, the transport of IgA and anticholera toxin IgA antibodies from the blood to the bile declines up to 6-fold between 3 and 25 months of age (9, 10). The hepatocellular translocation of vesicles containing endocytosed IgA and another glycoprotein, horseradish peroxidase, is significantly impaired, 60% and 40%, respectively, in rats pretreated with the microtubule depolymerizing agent, colchicine (3, 4). A similar decline (>40%) in the translocation of IgA-laden vesicles occurs in untreated rat hepatocytes as a function of aging alone (9). Microtubule integrity may be essential for the transport of newly synthesized IgA receptor (secretory component), a nonrecycled glycoprotein, to the hepatocyte plasma membrane (4). Interestingly, a 4-fold loss of IgA receptors from hepatocyte plasma membranes accompanies aging in the rat (11). Since endocytosed IgA does not get transported to the bile canaliculus in old rats, the number of IgA receptors is diminished in these animals, and colchicine elicits a similar decline in the hepatobiliary transport of IgA in young rats, we speculated that aging may be accompanied by microtubule dysfunction, e.g., reduced tubulin synthesis, altered tubulin polymer to monomer ratio that culminates in impaired cell function(s). Since microtubule associated proteins (MAPS) are essential to optimal microtubule function, changes in the quality

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or quantity of these components may also contribute to an age-related dissociation of the cytoskeleton. The present study describes results from a correlated structural and biochemical analysis of rat hepatocyte microtubules as a function of aging.

Methods

Animals. Male Fischer 344 (CDF) rats, aged 3–6 months (young), 12–16 months (mature), and 24–30 months (old; <50% survivorship), obtained from the National Institute on Aging colony (Harlan Industries, Inc., Indianapolis, IN), were used throughout the study. The animals were maintained under barrier conditions and fed laboratory chow and water *ad libitum* in accordance with the guidelines of the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources. All of the rats were subjected to a 12-hr fast prior to sacrifice to minimize the hepatic content of glycogen, which interferes with morphologic and biochemical analyses.

Electron Microscopic Analysis. Six young and six old animals were anesthetized with sodium pentobarbital, their livers were perfused via the hepatic portal vein with 2.7% glutaraldehyde and 0.8% paraformaldehyde in 0.2 *M* sodium bicarbonate, buffer, and the tissue was prepared for electron microscopic examination (12). Six tissue blocks, three from Zone 1 and three from Zone 3, were selected from each of the animals, and five electron micrographs per tissue block were taken and enlarged to a final magnification of ×45,000. The photographic fields were chosen according to an unbiased, systematic sampling procedure which required that the grid squares be covered with hepatocyte cytoplasm, two sides of the field be defined by grid bars, and the field be devoid of nuclear profiles. This sampling yielded 180 micrographs/age group and a total of 360 fields for analysis. Each micrograph was reviewed and every identifiable microtubule profile, regardless of length, was scored, as was each peroxisome in the same field. Peroxisomes were selected as the internal standard, since previous quantitative analysis demonstrated that the relative volume of this organelle compartment remains unchanged during aging in Fischer 344 rats (12). The data were expressed as the number of microtubule and peroxisome profiles per unit area of hepatocyte cytoplasm (five photographic fields) and as the ratio of microtubule to peroxisome profiles.

Quantitative Analysis of Tubulin and MAPS. Total and free tubulin as well as MAPS concentrations were determined in high-speed supernatants prepared from liver as described previously (13). Livers were homogenized in 2 vol (w/v) of Mes buffer (100 m*M* 2-[*N*-morpholino]ethanesulfonic acid, 0.5 m*M* $MgCl_2$, and 1.0 m*M* EGTA) with or without 4 *M* glycerol. Homogenates were centrifuged for 60 min at 100,000 *g* and the resultant supernatants were used to measure free (with glycerol) and total (without glycerol) tubulin.

Mouse monoclonal anti-α-tubulin and mouse monoclonal anti-β-tubulin antibodies of known specificities (Amersham) were used to quantitate α- and β-tubulin, respectively (14). MAP_1 content of the supernatants was measured using a monoclonal anti-MAP_1 antibody (clone HM-1; Sigma), which reacts with MAP_1 only. MAP_2 and tau were measured with a polyclonal rabbit anti-MAPS antibody (Sigma), which reacts only with these heat-stable MAPS. Biotinylated goat antimouse IgG and biotinylated goat antirabbit IgG were supplied by Cappel. The specificities of the antibodies were assessed by Western blot analysis (data not shown).

The relative amounts of α- and β-tubulin, MAP_1, MAP_2, and tau were determined by enzyme-linked immunosorbent assay. Microtiter plates were coated with serially diluted liver supernatants. Plates were incubated with a 1/1000 dilution of anti-α-tubulin, anti-β-tubulin, anti-MAP_1, or anti-MAPS mouse antibody. Wells were reacted with biotinylated goat antimouse IgG or biotinylated goat antirabbit IgG, avidin-conjugated horseradish peroxidase (Vector), and *ortho*-phenylenediamine (Zymed). The reaction product was stopped with 2 *N* sulfuric acid and the absorbance was measured at 490 nm. Since tubulin is a highly conserved protein, bovine brain tubulin purified according to the method of Sloboda *et al.* (15) was used as a tubulin standard. Standard curves were established for α- and β-tubulin using the corresponding monoclonal antibody. Liver tubulin values were calculated from the linear portion of the standard curves and are the result of the addition of α- and β-tubulin concentrations.

Results

A reassessment of stereologic and quantitative autoradiographic data generated in a previous study demonstrated that the translocation of ^{125}I-IgA-containing endocytic vesicles from the hepatocyte sinusoidal surface to the bile canaliculus is reduced by at least 40% in old versus young rats (9). This reduction reflects differences in vesicle movement or number rather than in ^{125}I-IgA autoradiographic grain density, since vesicles in young and old rats contained 29% and 26%, respectively, of the total grains counted in the hepatocytes.

Neither the morphologic appearance nor the relative concentration of hepatocyte microtubule profiles, i.e., the number per uniform area of hepatocyte cytoplasm, changed appreciably between 6 and 30 months of age (Table I). The microtubule profile densities estimated in young and old rats were within 10% of each other. The number of peroxisomes in the same photographic fields was similar in both age groups, i.e., within 15%. Furthermore, expression of the microtubule pro-

Table I. Effect of Aging on the Number of Rat Hepatocyte Microtubule and Peroxisome Profiles[a]

Age (mos)	Microtubules (no./unit area)[b]	Peroxisomes (no./unit area)	Microtubules/ peroxisomes
6	2.8 ± 1.9	13.3 ± 2.7	0.21
30	3.1 ± 1.8	11.1 ± 3.4	0.28

[a] Values represent the means of six rats (180 photographic fields) ± SD.
[b] Data expressed as the number of organelle profiles per unit area of hepatocyte cytoplasm; a unit area constitutes the cytoplasmic area included within five photographic fields at a final magnification of ×45,000.

files relative to the number of peroxisomes yielded similar ratios in young and old animals, 0.21 and 0.28, respectively.

On the other hand, biochemical analysis revealed several age-related changes in the concentrations of liver tubulin and MAPS (Table II). Increasing age is accompanied by a shift in the free to total (α and β) tubulin ratio, i.e., a 2-fold increase in mature and old animals in comparison to young rats. The free tubulin fraction constitutes 45% of the total tubulin pool in young rats, but this fraction increases to 85% and 88% in mature and old animals, respectively. This shift is not due to a unilateral increase in the free tubulin fraction, but appears to be a consequence of an increase in the free tubulin content in mature rats and a decline in the total tubulin content of old animals. The hepatic content of polymerized α- and β-tubulin exhibits a decline from 2.6 to 0.7 to 0.3 mg/g liver in 3-, 12-, and 24-month-old rats, respectively. While a concomitant change in the total MAP_1 concentration appears to be inconsequential, there is a >3-fold increase in the free fraction (Table II). A more pronounced shift occurs in the heat-stable MAPS, i.e., MAP_2 and tau. The concentration of total heat-stable MAPS declines >40%, whereas the corresponding free fraction(s) increases nearly 2-fold with increasing age.

Discussion

Circumstantial evidence from several laboratories, including our own, suggests that microtubule dysfunction accompanies aging (16; see Ref. 8 for a review). Quantitative electron microscopic autoradiographic data from a previous study suggested that the microtubule-dependent movement of IgA-containing vesicles from the sinusoidal surface of hepatocytes to the bile canalicular membrane was impaired in rats with increasing age (9). The distribution of radiolabeled IgA to endocytic vesicles was similar in young and old rats, thus eliminating differences in grain density as a possible explanation. While we cannot exclude the possibility of an age-related shift in the number of endocytic vesicles, the nonpericanalicular cytoplasmic compartments in both age groups contained equal numbers of autoradiographic grains, suggesting that the endosome population is reasonably stable during aging. An alternative explanation is that the nonpericanalicular vesicle pool is saturated in young and old animals and, thus, the difference in the pericanalicular cytoplasm is a reflection of reduced translocation alone.

On the basis of these data, a correlated structural and biochemical analysis appeared to be a logical approach to the question of whether or not aging compromises hepatic microtubules. The mean number of microtubule profiles per unit area of hepatocyte cytoplasm was reasonably consistent, regardless of animal age. Since the volume density of the peroxisome compartment is unaffected by aging in the Fischer rat, it provides an excellent internal standard to compare shifts in the density of other organelles, e.g., microtubules (12). The absence of a significant shift in the hepatocyte microtubule to peroxisome profile ratio suggests that if aging affects microtubule function, correlated morphologic changes are more subtle. This probably reflects the low frequency of microtubule profiles observed in both age groups and the relatively small sample of liver tissue examined.

The age-related decline in total tubulin concentrations, as well as the increase in the free to total tubulin ratio, may be indicative of fewer intact microtubules. A shift in the latter parameter may go undetected in a morphologic analysis, since microtubule length cannot

Table II. Effect of Aging on the Concentrations of Rat Liver Tubulin (α and β) and Microtubule-Associated Proteins (MAP_1 and Heat-Stable MAPS)[a]

Age (mos)	Tubulin[b]		MAP_1[c]		Heat-stable MAPS[c]	
	Total	Free	Total	Free	Total	Free
	(mg/g liver)		(Titer-1)		(Titer-1)	
3–4	4.7 ± 0.8	2.1 ± 0.5	9.5 ± 2.5	3.8 ± 1.0	22 ± 6	17 ± 5.2
12–14	4.8 ± 1.0	4.1 ± 2.5	6.0 ± 1.4	7.0 ± 1.3	18.5 ± 4.5	32 ± 0.5[d]
24–25	2.4 ± 0.3[e]	2.1 ± 0.4	8.8 ± 2.1	13 ± 1.9[d]	14.6 ± 0.6[d]	32 ± 0.5[d]

[a] Values reflect the means ± SD of four animals.
[b] α- and β-tubulin measured by enzyme-linked immunosorbent assay using bovine brain tubulin as standard.
[c] MAPS concentrations expressed as mean reciprocal titers ($Titer^{-1}$).
[d] Denotes significant difference versus young values ($P < 0.05$).
[e] Denotes significant difference versus young and mature values ($P < 0.05$).

be estimated accurately due to differences in organelle diameter relative to section thickness and the random orientation of the cytoskeletal elements in the plane of section. Small changes in this index may impair function, but remain morphologically undetectable. A diminution of microtubule number and length may compromise the vectorial movement of endocytic vesicles. Caron *et al.* (5) reported that very low doses of colcemid, e.g., <0.5 m*M*, markedly inhibited the processing of endocytosed ligands in cultured rat hepatocytes (5). These data suggest that the vesicle translocating function of microtubules is extremely sensitive to tubulin polymer levels.

The marked loss of polymerized tubulin between 3 and 12 months of age (75%), followed by an additional 12% decline between 12 and 24 months, correlates reasonably well with concomitant reductions of 85% and 3% in the hepatobiliary transport of IgA in mature and old rats, respectively (9). The large decline in mature rats seems to be due primarily to an increase in free or unpolymerized tubulin, whereas the later reduction in total tubulin content has only a minimal impact on polymerized tubulin. Clearly, aging appears to affect hepatic microtubules by two separate and sequential events, namely an increase in the free to total tubulin ratio and a decline in the total tubulin content, both of which contribute, albeit differentially, to a diminution of polymerized tubulin levels. The effect of aging on the free to total MAPS ratio is universal, i.e., a gradual, yet substantial, increase in MAP_1 and heat-stable MAPS (MAP_2 and tau). This shift to the free state may reflect a decrease in the affinity of MAPS for tubulin polymer and/or the concentration of polymerized tubulin. Factors that may contribute to reduced MAPS-tubulin binding include, but are not limited to, reduced dephosphorylation of MAPS or posttranslational modifications to polymerized tubulin (17). Age-related alterations in the capacity to phosphorylate or dephosphorylate a gene product are not without precedent (18). Furthermore, since MAPS directly affect monomer-polymer equilibrium by promoting tubulin assembly *in vitro* (14) and possibly *in vivo* (19), the loss of MAPS-tubulin affinity may also reduce the relative amount of tubulin assembled in old animals. Since we have shown that the *in situ* concentration of hepatocyte smooth-surfaced endoplasmic reticulum and the yield of hepatic microsomes decline substantially during aging in rats, we do not suspect that the age-related changes in polymerized tubulin and MAPS reflect concomitant differences in the binding of these moieties to membranes during isolation (20, 21). Since liver cells from old rats contain less membrane than those from young animals, we suggest that less, not more, tubulin or MAPS would be lost to recovery with increasing age. Our data lend credence to the supposition extended by Rao and Cohen (8) that aging may be accompanied by perturbations of microtubule function(s) which, in turn, contribute to the expression of biomarkers characteristic of normal aging (8). Subsequent studies will focus on the effects of aging on the synthesis and turnover of tubulin, as well as on its capacity to polymerize.

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