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Quantifying dense bodies and lipofuscin during aging: a morphologist's perspective

Douglas L. Schmucker [a,b,c,*], Howard Sachs [d,e]

[a] *Cell Biology & Aging Section (151E), San Francisco Veterans Affairs Medical Center, 4150 Clement Street, San Francisco, CA 94121, USA*
[b] *Liver Center, University of California, San Francisco, CA, USA*
[c] *Department of Anatomy, University of California, San Francisco, CA, USA*
[d] *Department of Anatomy and Neuroscience, Milton S. Hershey Medical Center, Pennsylvania State University, Hershey, PA, USA*
[e] *Office of the Dean, Pennsylvania State University, Harrisburg, PA, USA*

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Abstract

Secondary lysosomes, residual or dense bodies containing lipofuscin or age pigment accumulate in post-mitotic and inter-mitotic cells during aging. The consensus is that the accumulation of this auto-fluorescent material is an index of cellular senescence. Biochemical and morphological studies have independently demonstrated marked age-related increases in the cell and tissue contents of lipofuscin. Most morphological studies on aging have been qualitative, have included only two or three age groups and have not yielded data that are easily correlated with biochemical analyses. One of the best documented age-related changes in hepatocytes and cardiac myocytes is the accumulation of dense bodies and lipofuscin inclusions. Independent stereologic studies reported two- to eightfold age-related increases in the dense body volume fraction of rat hepatocytes. Furthermore, we reported a fourfold increase in the dense body volume fraction of cardiac myocytes in rats between 6 and 30 months of age. These and other studies confirm the use of quantitative morphology to estimate the increases in dense body and lipofuscin inclusions as indices of age. Whether or not the accumulated lipofuscin compromises cell functions in senescent animals has not been adequately addressed. On the one hand, there is little evidence that several-fold increases in this subcellular compartment impair the functional capacities of either hepatocytes or cardiac myocytes. On the other hand, the age-related accumulation of immunoprecipitable, but

* Corresponding author. Tel.: +1-415-221-4810x3450; fax: +1-415-750-6927.
E-mail address: coach@itsa.ucsf.edu (D.L. Schmucker).

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catalytically inactive, lysosomal enzymes in both liver and heart muscle may be a reflection of increased lipofuscin deposits in the dense bodies.

Keywords: Dense bodies; Lysosomes; Lipofuscin; Age pigment; Stereology; Cathepsin

1. Background

Secondary lysosomes and residual bodies, collectively referred to as dense bodies, containing lipofuscin or age pigment accumulate in post-mitotic and intermitotic cells during aging. The general consensus is that the accumulation of this autofluorescent material represents a universal index of cellular senescence. Numerous biochemical and morphological studies have independently demonstrated marked age-related increases in the cell and tissue contents of lipofuscin (see for review: Porta et al., 1995). Nevertheless, a number of questions pertaining to the intracellular nature or content of lipofuscin remain unresolved. For example, neither the origin nor the finite composition of lipofuscin has been clearly elucidated and unanimously accepted by investigators in this field. Furthermore, there are issues concerning the relationship of lipofuscin to organelles, whether or not the accumulated lipofuscin compromises the function(s) of these organelles and what type of measurement is the most valid for estimating intracellular lipofuscin content. There is a running controversy regarding the relative benefits of measuring lipofuscin content by biochemical methods following tissue extraction or by quantitative morphological procedures (Sheehy, 1996).

Although lipofuscin was initially described during the mid-19th century, it was not until the early 1950s that investigators demonstrated an age-related increase in the lipofuscin content of the human liver. This observation was followed by a series of studies, largely in the liver, that localized lipofuscin to lysosomes or dense bodies, described its appearance in the electron microscope and attempted to isolate and characterize the intracellular lipofuscin inclusions (for review, see Porta, 1991). The purpose of this retrospective analysis is to reevaluate morphological and biochemical data concerning lipofuscin accumulation during aging, the relationship of lipofuscin to dense bodies and the validity of using microscopy procedures to estimate the amount of intracellular lipofuscin.

2. Lipofuscin content of rat cardiac myocytes during aging

A number of studies have described an age-related accumulation of lipofuscin in cardiac myocytes in rodents and hamsters (Sachs et al., 1977; Porta et al., 1982; Ikeda et al., 1985a,b; Schmucker and Sachs, 1985). Electron microscopic analysis reveals several qualitative changes in the dense bodies as animals age, e.g. increases in the electron density of the dense body matrix and in the size of these inclusions, an accumulation of distinct electron lucid or lipid-like inclusions in the matrix (Fig. 1).

Quantitative microscopic procedures provide the means to estimate the relative volumes and surface areas of organelles and inclusions in situ. Using both fluorescence and electron microscopy, Porta demonstrated good agreement between the volume fractions of dense bodies and lipofuscin inclusions in rat cardiac myocytes during aging (Porta et al., 1982, 1995) (Fig. 2A). The volumes of both subcellular compartments increased substantially with age. Our own stereological analysis of rat heart muscle showed a much more gradual age-related increase in the dense body volume fraction (Schmucker and Sachs, 1985). Differences in animal strain, quantitative procedures and other variables may contribute to such discrepancies. Interestingly, when our results were expressed in a mode similar to that used by Travis and Travis (1972), i.e. the number of dense bodies per micrograph, there was excellent agreement between these two sets of data as evidenced by nearly equivalent correlation coefficients (Fig. 2B). Other studies have reported: (a) good agreement between the number of dense bodies per area of myocyte sarcoplasm and

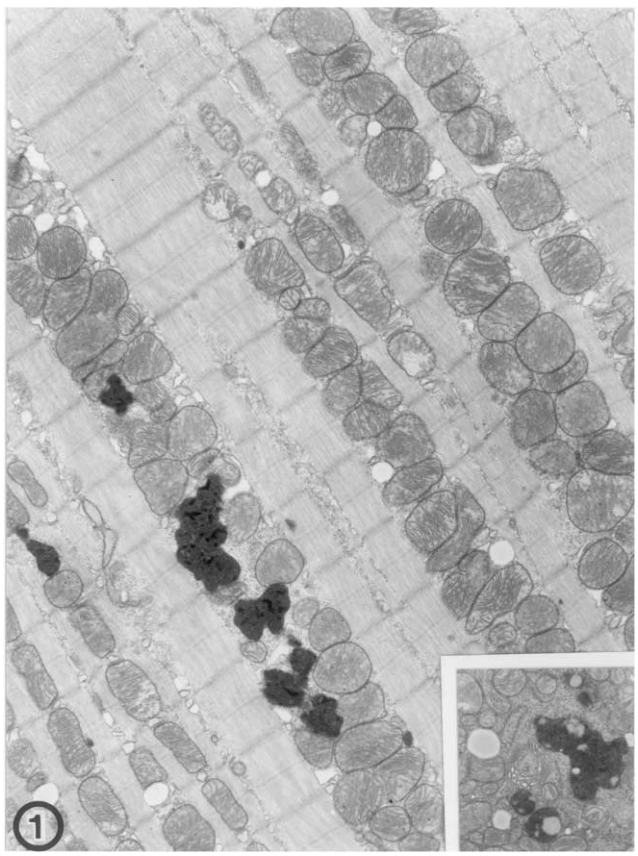

Fig. 1. Electron micrograph of cardiac myocytes from a senescent (30 months old) rat. The dense bodies are prominent (DB) with areas of electron lucidity in their matrix (inset), arrows. M, mitochondria; 15 000 × ; inset, 15 000 × .

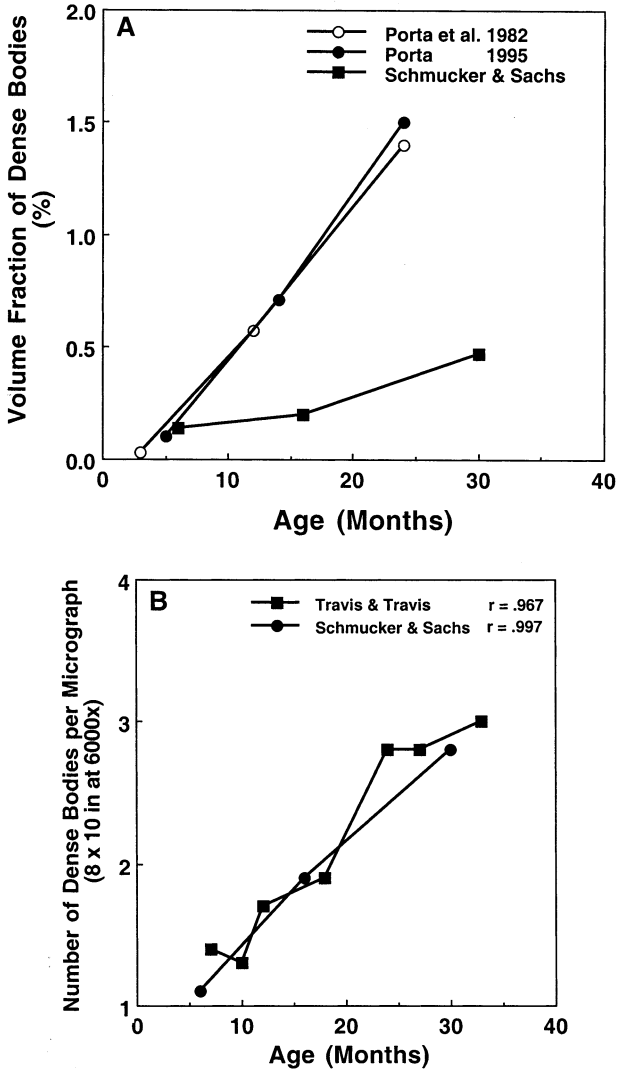

Fig. 2. (A) Volume fractions of dense bodies and lipofuscin inclusions in rat cardiac myocytes versus age. The age-related increases in the volume fractions of dense bodies (open circles) and lipofuscin inclusions (closed circles) reported by Porta (1991) and Porta et al. (1982) are identical. Schmucker and Sachs (1985) found an increase in the volume fraction of the dense body compartment in old Fischer rat cardiac myocytes in comparison to young animals; this parameter was much greater in the studies by Porta (1991) and Porta et al. (1982). (B) A comparison of the number of rat cardiac myocyte dense bodies per standard 8×10 in electron micrograph at similar magnifications during aging reported by Travis and Travis (1972) (squares) and Schmucker and Sachs (1985) (circles). These data are in good agreement as evidenced by the nearly identical correlation coefficients, i.e. $r = 0.967$ and 0.997.

the relative area of sarcoplasm occupied by lipofuscin inclusions and (b) age-related increases in both parameters (Ikeda et al., 1985a,b).

3. Lysosomal enzymes in cardiac muscle during aging

Current morphological evidence suggests a good correlation between dense bodies and lipofuscin inclusions in rat cardiac myocytes since the volume fractions of both subcellular compartments increase during aging. The suggestion that dense bodies and lipofuscin share, in part, a common subcellular compartment raises the question of whether or not the accumulation of lipofuscin compromises lysosomal function. Stereology affords the opportunity to compare organelle volumes and the activities of their intrinsic enzymes since both the volume fraction of a subcellular compartment and the activity of a constituent enzyme may be expressed per gram of tissue.

Acid phosphatase is a lysosomal marker enzyme used as an index of acid hydrolase activity in these organelles. Despite the marked increase in the volume fraction of the dense body compartment, our studies showed that the specific activity of acid phosphatase in rat cardiac myocytes remains unchanged during aging (Schmucker and Sachs, 1985) (Fig. 3A). However, lysosomes contain a wide array of enzymes and the activity of one does not dictate the activities of the other constituent enzymes. Therefore, we measured the activity of another lysosomal marker enzyme, β-glucuronidase. A comparison of the dense body volume fraction and β-glucuronidase activity in rat cardiac myocytes showed both parameters increasing linearly during aging, a pattern different from that of acid phosphatase (Schmucker and Sachs, 1985) (Fig. 3B).

Another family of lysosomal enzymes, the cysteine proteases, has been implicated in a hypothesis on the origin of lipofuscin. This hypothesis proposes that an age-related decline in the efficacy of certain cysteine proteases contributes to reduced degradation and turnover of effete intracellular proteins and culminates in the intracellular accumulation of lipofuscin (Ivy et al., 1984, 1991). Interestingly, the number of cathepsin B-positive myocytes in rat heart increases fourfold during aging, although an increase in the number of cathepsin B-positive cells does not necessarily connote a concomitant increase in either specific or total enzyme activity (Porta et al., 1995). The latter authors compared the volume fraction of lipofuscin inclusions in rat myocytes during aging with cathepsin B activity and reported a 50% decline in enzyme activity, an observation that lends credence to the cysteine protease hypothesis (Porta et al., 1995).

One possible explanation for the diminished activity of cysteine proteinases during aging may be found in a study by Wiederanders and Oelke (1984). These investigators measured age-related changes in the concentration of another cathepsin isoform, cathepsin D, in rat cardiac muscle using both an immunoprecipitation and an enzymatic method. The amount of immunoprecipitable cathepsin D increased linearly across the age span studied (Fig. 4). However, the amount of enzyme estimated on the basis of catalytic activity declined substantially during the

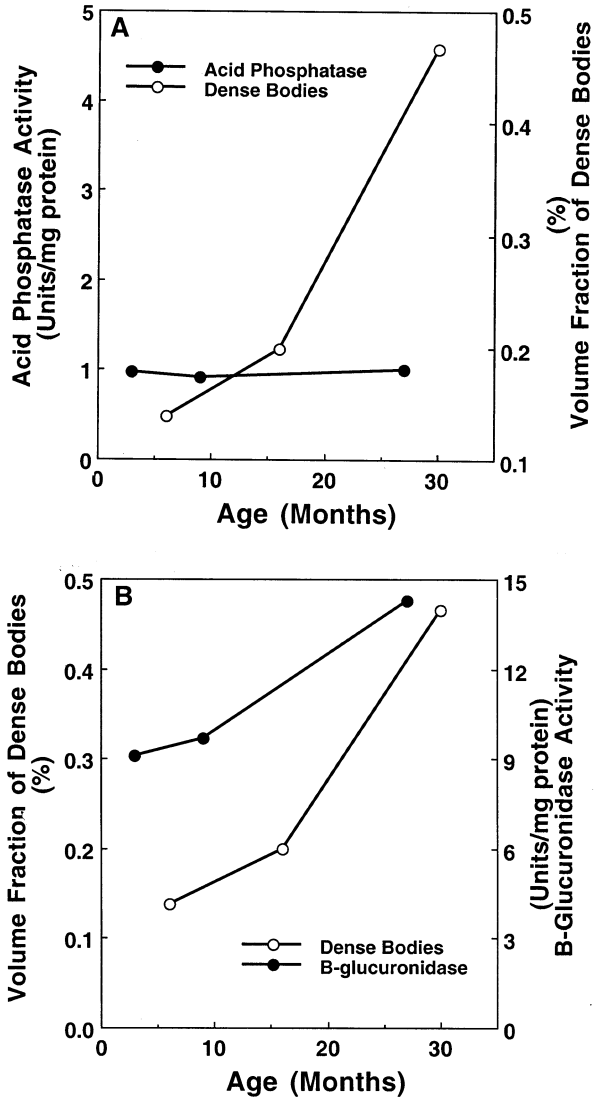

Fig. 3. (A) Comparison of acid phosphatase activity and dense body volume fractions in cardiac myocytes as a function of age in Fischer 344 rats. The activity of the lysosomal enzyme, acid phosphatase, remains unchanged (closed circles) despite the marked increase in the size of the dense body compartment (open circles). (B) A similar comparison of dense body volume fraction and the lysosomal enzyme β-glucuronidase in rat cardiac myocytes versus animal age. β-Glucuronidase activity increases in parallel with the age-related increase in the dense body volume fraction (data derived from Schmucker and Sachs, 1985).

latter half of the lifespan, i.e. to the extent that the level measured in senescent rats was nearly equivalent to that found in immature animals. One interpretation is that immunoprecipitable, but catalytically effete, cathepsin D accumulates in cardiac myocytes as the animals age, thus diminishing the absolute activity, i.e. activity per μg of enzyme protein. Similar age-related accumulations of immunoprecipitable, yet inactive, enzymes have been reported in several tissues. For example, we showed that immunoprecipitable, inactive microsomal NADPH cytochrome P450 reductase accumulates in the livers of senescent male rats and may contribute to the well documented decline in hepatic Phase I drug clearance (Schmucker and Wang, 1983). The age-related accumulation of effete enzymes may reflect reduced turnover or increased post-translational modifications in these proteins.

4. Lipofuscin content of rat hepatocytes during aging

Certain cell types that exhibit low rates of mitosis or, at least, retain the potential for division (intermitotic) may also accumulate lipofuscin during aging, e.g. hepatocytes. The fact that dense bodies and lipofuscin increase with age in hepatocytes is well-established (see Schmucker, 1990 for a review). Hepatocyte dense bodies undergo both qualitative and quantitative age-related changes similar to those observed in cardiac myocytes (Fig. 5).

Our comprehensive stereological analysis of the male Fischer 344 rat liver revealed a rapid increase in the dense body volume fraction during the first 20 months of life, followed by a decline in the size of this subcellular compartment during the third decade (Fig. 6) (Schmucker et al., 1978). These shifts in the volume

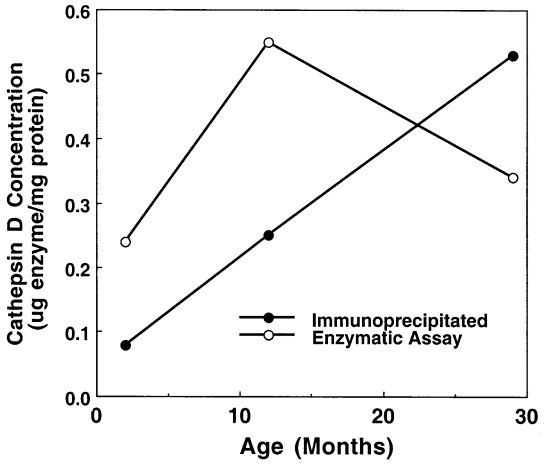

Fig. 4. Concentrations of cathepsin D in rat cardiac myocytes versus animal age measured by an immunoprecipitation method (closed circles) and an enzymatic assay (open circles). These data suggest an age-related accumulation of immunoprecipitable cathepsin D with diminishing catalytic activity (data derived from Wiederanders and Oelke, 1984).

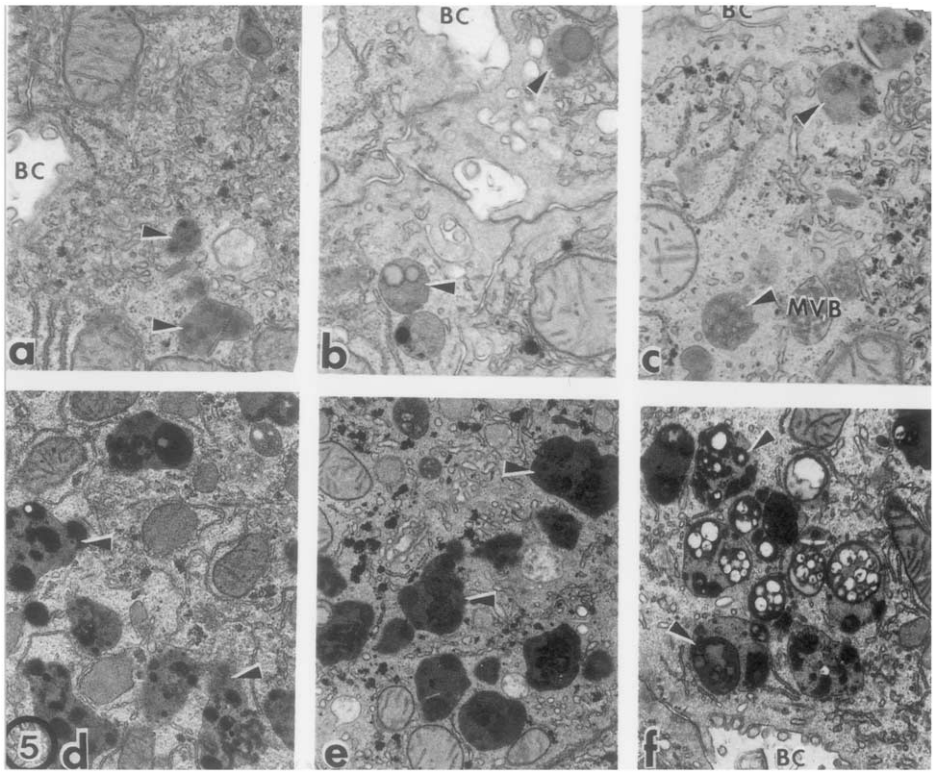

Fig. 5. Series of electron micrographs demonstrating the morphological alterations in hepatocyte dense bodies during aging in rats: (a) 1 month; (b) 10 months; (c) 16 months; (d) 25 months and (e, f) 30 months. Dense bodies exhibit typical appearance with areas of electron density or lucidity in rats up to and including 20 months of age (a–c). By the time the animals reach 25 months of age, the dense bodies are markedly more numerous and electron dense (d). This transition continues through 30 months (e) and, ultimately, much of the electron density is replaced by areas of electron lucidity in the dense body matrix (f). The sequential changes in electron density may reflect age-related accumulations of lipofuscin; 15 000 × (data derived from Schmucker, 1990).

fraction of this organelle occurred both in zones 1 and 3 of the hepatic lobule. Stereological data from several different laboratories substantiate significant and non-uniform age-related increases in the number and volume fraction of dense bodies across the liver lobule in rodents (Knook and Sleyster, 1976; Meihuizen and Blansjaar, 1980; De Priester et al., 1984; David, 1985). The consensus is that the dense body compartment increases with age in rats, but at different rates in different strains.

A comparison of the data of Ikeda et al. (1985a,b) and Iwasaki et al. (1988) yields an interesting result. These investigators measured the numbers of dense bodies or lipofuscin inclusions in rat hepatocytes as a function of age. As anticipated, Ikeda et al. (1985a,b) reported that the number of dense bodies per 100 μm^2 of cytoplasm increased linearly during aging. However, Iwasaki et al. (1988) found

that the number of lipofuscin inclusions remained unchanged for nearly two-thirds of the lifespan before increasing during senescence. These data suggest that the dense bodies observed during the first 20 months of the lifespan may be devoid of lipofuscin, despite the age-related increases in the number and volume fraction of this organelle.

5. Lysosomal enzymes in rat hepatocytes during aging

We previously reported differential responses of two rat liver lysosomal enzymes, acid phosphatase and β-glucuronidase, to aging (Schmucker and Wang, 1979). Acid phosphatase activity remains virtually unchanged throughout the lifespan, whereas the activity of β-glucuronidase exhibits a moderate increase. De Priester et al. (1984) made an interesting observation upon measuring the volume fractions of the dense body and acid phosphatase-positive inclusion compartments in rat hepatocytes during aging. Although the rates at which the volume fractions of these two subcellular compartments increased were similar, the size of the acid phosphatase-positive compartment was three- to fivefold larger than that measured for the dense bodies. It appears that standard stereological analysis failed to detect a substantial number of inclusions with acid phosphatase activity. This discrepancy may reflect the inability to resolve primary lysosomes prior to their fusion with sequestration vacuoles at the magnification employed. Nevertheless, these small organelles do not contain lipofuscin and, therefore, they may not exhibit any correlation with lipofuscin inclusions.

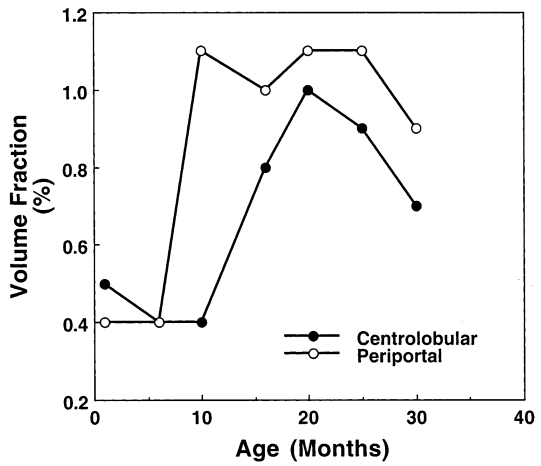

Fig. 6. Effects of aging and sublobular location on hepatocyte dense body volume fraction in rats. The dense body volume fraction exhibits dramatic increases in lobular zones 1 and 3 by 10 and 16 months of age, respectively. For much of the lifespan, the dense body volume fraction is greatest in the periportal hepatocytes (zone 1) in comparison to centrolobular or zone 3 cells (data derived from Schmucker et al., 1978).

As in rat cardiac myocytes, the response of different liver lysosomal enzymes to aging is variable, e.g. acid phosphatase and β-glucuronidase. However, depending on the study, the activity of hepatic cathepsin D remains unchanged, increases moderately or exhibits a marked increase as the animals age (Knook and Sleyster, 1976; Wiederanders and Oelke, 1984; Mertens-Strijthagen and De Schryver, 1989). Wiederanders and Oelke (1984) also measured the concentration of cathepsin D in the rat liver using both immunoprecipitation and enzymatic procedures. As in rat cardiac myocytes, there occurs a rapid accumulation of immunoprecipitable enzyme between 10 and 30 months of age. This is accompanied by a much more gradual increase in the catalytic activity of this enzyme. This difference culminates in a disparity in the amount of cathepsin D measured by the two independent methods and suggests an age-related accumulation of immunoprecipitated enzyme expressing reduced catalytic properties.

6. Conclusions

Rat cardiac myocytes and hepatocytes accumulate dense bodies and lipofuscin inclusions during aging. Data from most studies suggest that the volume fractions of these subcellular compartments increase in parallel, at least during senescence. On the basis of the evidence presented, it is reasonable to consider the accumulation of dense bodies and lipofuscin inclusions as indices of aging in mammalian post-mitotic and inter-mitotic cells. Furthermore, quantitative microscopy (e.g. light and electron microscopic stereology, quantitative fluorescence microscopy) provides consistent and reproducible estimates of changes in these cellular constituents that occur as a consequence of aging. However, the role of increased lipofuscin deposits in effecting age-related changes in cell functions or other parameters, e.g. the accumulation of effete lysosomal enzymes, remains unresolved.

Acknowledgements

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7. Floor debate of the lecture of Dr. Schmucker

Zs.-Nagy: Dr. Schmucker, you mentioned that there are differences between the immunological detectable enzyme concentrations and enzyme activities. This is an old story that goes back to some 35 years ago. It was shown at that time that enzymes are present in inactive form or partly inactivated form, but which are still reacting immunologically. And I would like to emphasize another aspect of the story, that in vivo, in situ, the enzymes lose even more of their activities because of progressive drying out of tissues with age. The increase in dryness or loss of water

content is enough to cause an order of magnitude drop in the enzymatic activities. So it means that if you expressed enzyme activity per mg of total protein, this is a totally meaningless parameter, because you have to know how many enzyme molecules are present in the system (i.e., activity should be given per mole of enzyme). You have tremendous drop just simply due to increased physical density. These things are almost never considered in aging research.

Schmucker: I agree with Dr. Zs-Nagy that there is a problem with enzyme expression. You may recall that when we published our data on P-450 reductase, we made the point that is very important to express enzyme activity as absolute activity as opposed to specific activity. By absolute I mean catalytic activity per milligram of enzyme protein. The point I was trying to make about the cysteine proteases is that of a devil's advocate because I know the controversies surrounding Gwen Ivy's concept that the decline of the enzyme, and thus the decline in proteolysis, results in the accumulation of lipofuscin. In fact you can show age-related increases in specific enzyme activities (e.g., Dr. Porta's studies on cathepsin B in rat brain and heart). Several studies have shown increases in the immunoprecipitated activity of cathepsin D, but a decline in specific activity. These data support the concept of reduced lysosomal turnover as cells become older.

Zs.-Nagy: Since you quoted Ivy's protease inhibitor model, I want to say that I believe that there is a protein inhibition but no particular inhibitor factor is present. Just the physico-chemical density of the system is responsible. And this is enough to explain the decreased proteolysis because it slows down everything. So, the enzyme activities that you can measure in the test tube may increase or decrease as expressed by unit of protein, but in situ, if the same amount of enzyme is present, the activity slows down. The performance of a given enzyme in the higher physical density is lower, and this is perfectly enough to block the lysosomal system.

Palmer: Dr. Schmucker, does anybody ever shown the half-life of the age-pigment. For example, was anybody able to radiolabel the retina and come back a year later and see if there is a longer half-life for the proteins or other components of the residual bodies compared with the rest of the rat.

Schmucker: I don't know if I am the right person to answer this question. I think it is facetious to say that I have studied the turnover rates of certain proteins as a function of age and found that some of them have extended half-life, which I think simply reflects reduced turnover. The longer these proteins are exposed in situ to the deleterious influences or impacts of aging, the greater the possiblility for posttranslational modifications. But as far lipofuscin is concerned, I will defer your question to more experienced colleagues.

Katz: This will be the topic of one of the tomorrow's sessions during which the question of lipofuscin turnover will be addressed.

Porta: The whole question of the best way to express enzyme activity changes in relation to age continues being a controversial problem and the literature is still plagued with very conflicting results on the activities of lysosomal enzymes even when expressed in similar ways. In regard to the age-related changes in the activities of lysosomal enzymes in relation to the well known age-dependent increases of neuronal lipofuscin, it is important to recognise that different areas of the central nervous system, particularly in the brain, may show substantial differences in lysosomal enzyme activity and the same is true of the regional amounts of lipofuscin. I want to remind that our published histochemical data on cathepsin B was performed exclusively in the cortical neurons of the temporo-parietal areas of the brain, the same areas where lipofuscin was also morphometrically determined. I want also to refer to the extensive studies of Nakanishi et al. (Exp. Neurol. 126, 119–28, 1994) on the age-related changes in activities and localizations of cathepsins D, B, and L in the cerebal cortex, hippocompus, neostriatum and cerelbellum of young and aged rats. The data derived from these studies expressed as enzyme units per mg of protein showed that cathepsin D increased with age in all the brain regions studied, and that with the exception of the hippocampus, cathepsin B also increased in all the other areas. On the contrary, cathepsin L decreased with age in all the regions studied. I think that we have to be careful before enbracing a hypothesis on lipofuscino-genesis or on lipofuscin degradation on the basis of insufficient or non-existing direct supporting data like the hypothesis of lvy or the interpretation of this hypothesis by Dr. Zs.-Nagy.

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