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Original Contribution

Lipofuscin is formed independently of macroautophagy and lysosomal activity in stress-induced prematurely senescent human fibroblasts

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ABSTRACT

In the current literature, the lysosomal system is considered to be involved in the intracellular formation and accumulation of lipofuscin, a highly oxidized and covalently cross-linked aggregate of proteins that fills the lysosomal volume during aging. In contrast, our experimental results presented here suggest that both the autophagosomes and the lysosomal system are not mandatory for the formation of lipofuscin, since that material accumulates in the cytosolic volume if autophagy or lysosomal activity is inhibited. However, such an inhibition is accompanied by an enhanced toxicity of the formed protein aggregates. Furthermore, it could be proven that macroautophagy is responsible for the uptake of lipofuscin into the lysosomes.

Introduction

The intracellular accumulation of lipofuscin is one of the limiting factors in postmitotic cell aging. Understanding the basic mechanisms of lipofuscin formation and accumulation can contribute to ensuring the quality of life in old age and may identify ways to reduce the accumulation rate of protein aggregates in cells. Mammalian cells have several mechanisms for removing proteins when they are misfolded, become oxidized, or are damaged. One of the main degradation systems is the proteasome, a multisubunit protease. However, the proteasome only degrades unfolded monomeric proteins, so it cannot remove covalently cross-linked proteins from the cytosol [1,2].

The other basic degradation system is autophagy, which includes macro-, micro-, and chaperone-mediated autophagy. Macroautophagy functions as a stress response which is upregulated by oxidative stress, starvation, or other adverse conditions and is controlled by a specific set of *ATG* genes and their protein products (Atg). One of the discussed options for disposal of aggregated proteins is the uptake into the lysosomes via macroautophagy. In 2007 the term "aggrephagy" was introduced [3]. This form of macroautophagy seems to be relevant for the uptake of large protein aggregates or lipofuscin particles, which were released into the cytosol due to rupture of lysosomes.

The hallmark of macroautophagy is the formation of double-membrane vesicles called autophagosomes that sequesters portions of the cells cytoplasm and delivers them to the lysosome. The formation of autophagosomes in mammalian cells requires two processes: Atg12 conjugation and LC3 (microtubule-associated-protein-light-chain-3) modification. Atg12 is ligated to Atg5 in a process assisted by the enzymes Atg7 and Atg10. The Atg12–Atg5 complex then forms larger oligomers with Atg16. This Atg12–Atg5–Atg16 complex is essential for elongation of the isolation membranes and a key regulator of the autophagic process [4]. LC3 also assists autophagosome formation, possibly by enhancing membrane fusion [5]. The soluble cytosolic LC3I becomes ligated to the lipid phosphatidyl ethanolamine in reactions assisted by Atg4, Atg7, and Atg3. The lipidated form is called LC3II and anchors to autophagosomal membranes.

While the role of proteasome inhibition as a cause of age-related increases in protein oxidation and increased lipofuscin amounts is established, the contribution of the lysosomal system and the role of macroautophagy are less clear. Genetically determined disorders of lysosomal degradation lead to severe

Abbreviations: ATG/Atg, autophagy-related gene; ATP6V0A1, encoding gene of isoform a1 of vacuolar H^+-ATase V_0 domain; H_2DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; LAMP1, lysosomal-associated membrane protein 1; LC3, microtubule-associated-protein-light-chain-3; LF, lipofuscin; MDC, monodansylcadaverine; MEFs, mouse embryonic fibroblasts; NH_4Cl, ammonium chloride; PQ, paraquat; SIPS, stress-induced premature senescence.

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defects with an extreme overload of cells with undegraded material [6], but also during aging a progressive decline of lysosomal function is postulated. Age-related impairments in lysosomal proteolysis including dysfunctional regulation of pH, impaired lysosomal stability, and targeting of proteins are observed in many cell types [7,8]. So, over time defective proteins and organelles as well as lipofuscin slowly but progressive accumulate especially in postmitotic aging cells.

Our aim was to investigate the role of macroautophagy and the lysosomal system during aging in the formation and accumulation of lipofuscin. We used ATP6V0A1 siRNA for blocking of the lysosomal proton pump to interfere with the functionality of the lysosomes in an established "stress-induced premature senescence" (SIPS) model of human fibroblasts. SIPS is a model for inducing characteristic features of aged cells in young or "middle-aged" cells by application of chronic oxidative stress [9]. In a next step we inhibited autophagic sequestration of protein aggregates and performed further experiments with a mouse embryonic fibroblast (MEF) ATG5$^{-/-}$ cell line and human dermal fibroblasts, treated with ATG5 siRNA.

Materials and methods

Reagents

All chemicals were obtained from Sigma (Deisenhofen, Germany). Cell culture materials were purchased from Biochrom (Berlin, Germany) unless otherwise indicated.

Cell culture

Experiments were performed using human dermal fibroblasts obtained from skin tissue. The fibroblasts were a kind gift of Prof. Scharffetter-Kochanek (University of Ulm, Germany). Informed consent of the donor was achieved according to international rules. Fibroblasts were cultured in DMEM supplemented with 10% fetal calf serum (FCS), and 1% Glutamax. Mouse embryonic fibroblasts from WT and ATG5$^{-/-}$ embryos [10] were obtained from the RIKEN BRC cell bank (Tsubuka, Ibaraki, Japan) and maintained in DMEM with 10% FCS.

RNA interference

Pooled small interfering RNA (siRNA) oligonucleotides against ATG5 and ATP6V0A1, as well as a control siRNA (nontargeting pool), were purchased from Thermo Scientific Dharmacon (Lafayette, CO, USA). Efficacy and specificity of knockdown were assessed by immunoblotting. Cells were transfected at 70% confluence with 50 nM of the pooled oligonucleotide mixture by using Dharmafect transfection reagent following the manufacturer's protocols. After removal of the transfection media cells were allowed to recover for 10 h before further treatment.

Stress-induced premature senescence (SIPS)

SIPS was achieved by paraquat (PQ) treatment. The explicit procedure is described in Ref. [11]. PQ was applied to confluent cells for 10 days daily (3.0 μM per 10^6 cells). For further treatment (transfection, concanamycin A) PQ-pretreated cells were trypsinized and seeded subconfluent at Day 5 or 7 of PQ treatment.

Immunoblot analysis

Cells were lysed at 4 °C using 10 mM Tris-HCl (pH 7.5) buffer containing 1 mM Pefabloc, 0.9% Nonidet P-40, 0.1% SDS. The protein concentrations of the supernatants were determined according to the Lowry method. The amount of 15–30 μg of total protein in reducing Laemmli buffer (0.25 M Tris (pH 6.8), 8% SDS, 40% glycerol, 0.03% Orange G) was denatured at 95 °C for 5 min and applied to SDS-PAGE of 12% (w/v) acrylamide followed by electrophoresis and blotting onto nitrocellulose membrane according to standard procedures.

Immunodetections were performed with the following antibodies at dilutions recommended by the suppliers: mouse monoclonal LC3 antibody, mouse monoclonal Atg5 antibody (Nano Tools, Teningen, Germany), mouse monoclonal LAMP1 antibody, rabbit polyclonal GAPDH antibody, mouse monoclonal p62/SQSTM1 antibody, and rabbit polyclonal ATP6V0A1 antibody (ABCAM, Cambridge, UK). The blocking buffer and fluorescent-conjugated secondary antibodies were purchased from Li-Cor Biosciences (Lincoln, NE) and used according to protocols supplied by the manufacturer. The membranes were scanned and stained bands were quantified using an Odyssey Infrared Imaging System (Li-Cor Biosciences) according to the manufacturer's instructions.

Determination of cell viability using MTT

Cell viability was determined using a standard tetrazolium salt assay [12]. The absorbance was measured at 590 nm with a microplate reader (Synergy 2, BioTek, Bad Friedrichshall, Germany).

Measurement of autophagic acitivity using MDC

Cells were incubated with 0.05 mM MDC at 37 °C for 10 min [13]. Afterward cells were washed three times with PBS and collected in 10 mM Tris-HCl, pH 8, containing 0.1% Triton X-100. Intracellular MDC was measured using a fluorescence reader (Synergy 2, BioTek) at 360 nm excitation/530 nm emission. For normalization to the number of cells present in each well, DNA was stained with ethidium bromide at a final concentration of 0.2 μM and the DNA fluorescence was measured at 530 nm excitation/590 nm emission.

Visualization of MDC-stained autophagosomes and autolysosomes

Cells were incubated with 0.05 mM MDC at 37 °C for 20 min. After incubation cells were washed three times with PBS and fluorescence was microscopically investigated (Zeiss Axiovert 100 M running standard software, Zeiss, Jena, Germany) using an excitation filter of 360 nm and an emission filter of LP 420 nm.

Lipofuscin detection

Lipofuscin was detected via its autofluorescence using fluorescence microscopy with an excitation wavelength of 360 nm and an emission filter of 420 nm.

Visualization of the lysosomal system and its colocalization with lipofuscin

Lysosomes were labeled using "LysoTracker Blue" (Invitrogen, Karlsruhe, Germany) and according to the manufacturer's instructions. After bleaching away the LysoTracker signal the autofluorescence of lipofuscin remained. The detailed procedure can be found in [14].