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Reduced autophagy leads to an impaired ferritin turnover in senescent fibroblasts

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ABSTRACT

Changes in the two main intracellular degradation systems, the Ubiquitin-Proteasome System and the Autophagy-Lysosome pathway (ALP) are widely discussed as a hallmark of the aging process. To follow the age-related behavior of both degradation systems we examined their impact on ferritin, known to be degradable by both. Ferritin H was analyzed in young and senescent human fibroblasts, revealing a higher steady-state level in the senescent cells. By blocking both proteolytic systems, we confirmed that particularly the ALP plays a crucial role in ferritin H turnover. However, an unexpected increase in lysosomal activity in the senescent cells, suggests a dysregulation in the autophagy pathway. To further investigate the impaired ferritin H turnover, confocal microscopic colocalization studies of ferritin H with lysosomal-associated membrane protein 2a (Lamp2a) and monodansylcadaverine (MDC) were performed and clearly revealed the degradation of ferritin by macroautophagy. By induction of autophagy via inhibition of mTOR using rapamycin an increase of ferritin H turnover was obtained in senescent cells, demonstrating a mTOR dependent reduction of autophagy in senescent human fibroblasts.

1. Introduction

Degradation of modified and damaged proteins is an essential process for maintaining cellular functions and viability. It is known that several repair and antioxidant mechanism gradually decline during the aging process [1,2]. Furthermore, it is assumed that the production of reactive oxygen species, which varies greatly during different stages of life [3–5], additionally aggravate the decline of cellular defense mechanisms, such as catalase, glutathione peroxidase [6] and superoxide dismutase [7,8]. Dysfunction of regular proteolysis leads to increased accumulation of oxidized proteins and serious functional consequences, since proteins act as receptors, transcription factors and enzymes. In addition, these modified and damaged proteins can cross-link and form protein aggregates, involved in many age-related diseases [9]. For intracellular protein turnover cells possess two major proteolytic systems: the Ubiquitin-Proteasome System (UPS) and the Autophagy-Lysosome pathway (ALP) [10]. Among its various functions, a major role of the UPS, more precisely the 20S proteasome, is the degradation of unfolded, oxidized and short-lived proteins, preventing their accumulation [11–13]. Additionally, the 26S proteasome of the UPS is able to specifically degrade ubiquitin-labeled proteins, demonstrating the selectivity of the UPS [14]. The ALP involves the engulfment of cytoplasmic material, such as organelles or long-lived proteins and the delivery of sequestered cargo into the lysosomes followed by their degradation. For the transfer to the lysosomes three different types of autophagy are well described. One is the chaperone-mediated autophagy (CMA) responsible for the specific delivery of soluble cytosolic proteins. Additionally to CMA, two other types of autophagy are known, microautophagy and macroautophagy. During macroautophagy a *de novo*- membrane is formed which further extend to a double-membrane vesicle, the so-called autophagosome. The autophagosome then fuses with the lysosome to form the autolysosome, where the degradation of the cell constituents by different cathepsins takes places. In order to clarify the degradation of ferritin by macroautophagy, colocalization of the protein with autophagosomes, via MDC, or Lamp2a, indicating an involvement of CMA, can

Abbreviations: Lamp1, Lysosomal-associated membrane protein 1; ConA, Concanamycin A; LC, Lactacystin. DTT, Dithiothreitol; LC3, Microtubule-associated protein 1A/1B-light chain 3; mTOR, mammalian target of rapamycin; ATGs, Autophagy-related proteins; ALP, Autophagy-Lysosome pathway; UPS, Ubiquitin-Proteasome System

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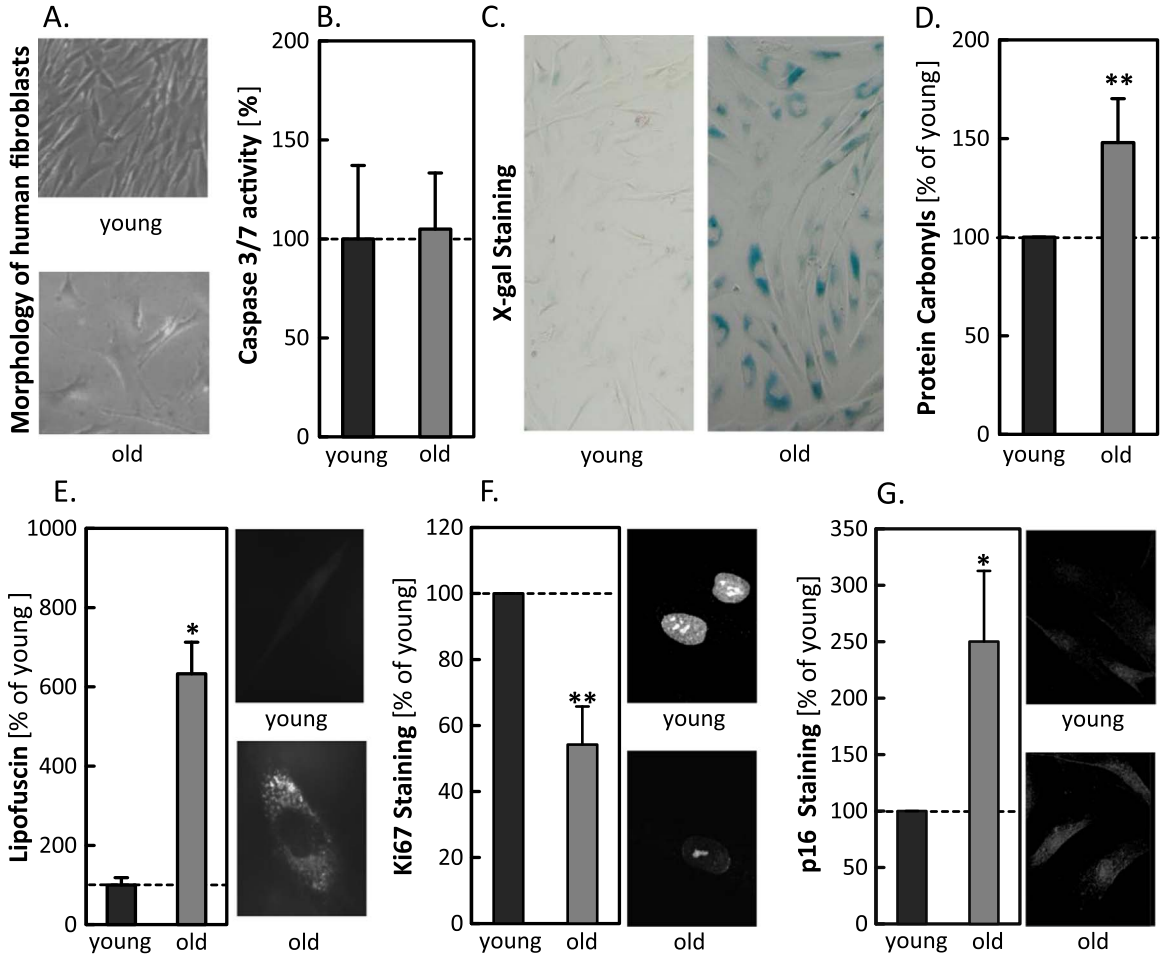

Fig. 1. Determination of senescence parameters in human fibroblasts. To verify senescence of the old fibroblasts, the following senescence-associated parameters were determined: (A) differences in morphology by transmitted light microscopy, (B) levels of caspase 3/7 activity, (C) X-gal staining for senescence associated β-galactosidase (70.8% of old cells are positive for SA-β-Gal) (D) quantification of protein carbonyls in young and old cells and (E) quantitative analysis of fluorescent aging pigment lipofuscin, (F) Ki67 and (G) p16 staining. Data for (D) were normalized to Ponceau S staining and shown in percentages to the young control. The data represent the mean ± SD of at least three independent experiments. In general, statistical significant differences between young and old cells were evaluated by student's *t*-test and are shown by *$p < 0.05$ and **$p < 0.01$ compared to the young.

be performed. Furthermore, macroautophagy has been described to be negatively regulated by mammalian target of rapamycin 1 (mTORC1) [15]. One way to verify that autophagy is responsible for ferritin turnover is the inhibition of mTOR, especially mTORC1 (referred to as mTOR from here on). It is well-known that mTOR can suppress autophagy and that its inhibition via rapamycin leads to an induction of autophagy [16,17]. To directly study the age-related changes in intracellular protein turnover we used a cytosolic protein, ferritin H, which is known to be degraded by both proteolytic systems, the UPS [18,19] and ALP [20,21]. In mammalian cells the cytosolic iron storage protein ferritin plays a crucial role in iron metabolism. It is composed of 24 protein subunits, which are divided into two isoforms, ferritin light chains and ferritin heavy chains. The ferroxidase activity of ferritin H oxidizes free Fe^{2+} to Fe^{3+} and thus stores it safely. In the event of cellular iron deficiency, iron can be released from ferritin again. In the presence of reactive oxygen species, such as superoxide and hydrogen peroxide, free iron can catalyze highly reactive hydroxyl radicals via the Fenton reaction, which are able to damage lipids, proteins and DNA. Therefore, a strict regulation of iron uptake, storage and release is essential for maintaining cellular homeostasis. Hence turnover of ferritin H can bring further information in the changes and compensatory mechanisms of the proteolytic systems during aging and on the other hand it will give further insight in the ferritin H turnover. While, recent reports already described a more specific degradation of ferritin H via autophagy in knock-out and knock-down systems [22], this study compares the changes of ferritin turnover in young and senescent cells, demonstrating that reduced autophagy is responsible for impaired ferritin turnover in senescent human fibroblasts.

2. Results

2.1. Protein and mRNA expression of ferritin H in young and in vitro aged fibroblasts

To monitor the age-related changes in the proteolytic systems we performed all subsequent experiments in young (up to PD20) and senescent fibroblasts (up to PD60, referred to as old from here on), an aging model already described in [23]. To confirm cellular senescence we analyzed morphological differences, x-Gal staining, where 70.8% of the old cells were positive for SA-β-Gal, formation of protein carbonyls [24], lipofuscin accumulation [25], Ki67 and p16 (Fig. 1). The combination of all these parameters clearly demonstrates the senescence state of the old fibroblasts, necessary for all ongoing experiments. To show that apoptosis is not affecting our senescence model, we additionally determined caspase-3/7 activity in young and old cells (Fig. 1B). By analyzing the ferritin H amount in fibroblasts with different population doublings we interestingly monitored significant higher basal levels of the protein via immunoblot (Fig. 2A) and fluorescence microscopy (Fig. 2B) in the old cells. To examine whether the higher quantity of ferritin H results from an increased protein