Oxidative stress plays a crucial role in the development of the aging process and age dependent diseases. Both are closely connected to disturbances of proteostasis by protein oxidation and an impairment of the proteasomal system. The final consequence is the accumulation of highly cross-linked undegradable aggregates such as lipofuscin. These aggregates of damaged proteins are detrimental to normal cell functions. Here we provide an overview about effect of these aggregates on the proteasomal system, followed by transcription factor activation and loss of cell viability. Furthermore, findings on the mechanism of radical genesis, proteasomal inhibition and the required components of lipofuscin formation were resumed.

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Introduction

In unstressed situations protein homeostasis is balanced by folding and stabilization of proteins by chaperones of the Hsp family [1] and the controlled degradation of proteins by the proteasomal system. The proteasome exists in different forms, and its activity is modulated by multiple regulators. The 20S core proteasome contains the proteolytic activity and selectively degrades a multitude of oxidized proteins [2–5], as well as other substrates [6], in an ubiquitin- and ATP-independent manner. When the core 20S proteasome combines with two 19S regulators, the 26S proteasome is formed [7] which selectively removes polyubiquitinated proteins [8–10]. Under stress conditions and, therefore, most notably during aging the balance between protein damage and clearance of damaged proteins is disturbed leading to a malfunctioning of proteostasis and an accumulating mass of oxidized proteins, aggregate and aggresome formation and finally to the accumulation of highly cross-linked materials such as lipofuscin, compromising cell viability. Accumulation of aggregates in postmitotic cells seems to be especially dramatic, since they are not able to dilute this material by cell division.

Previous work has shown that especially the 26S proteasome is highly susceptible to inactivation during oxidative stress [11,12] and although inhibition of 26S proteasomes could be caused by oxidation products such as protein aggregates, it is most likely a stress-triggered disassembly [13,14] (Fig. 1A). It could be suspected, that this disassembly of 26S proteasomes serves to increase 20S proteasome abundance, allowing cells to clear irreparably damaged proteins more effectively [9,15,16]. But inhibition of 26S is accompanied with the accumulation of undegraded, polyubiquitinated proteins, which are sensed by the ubiquitinbinding domain of HDAC6 (Histone deacetylase 6) [17].

Via HDAC6 this proteotoxic stress acts as a signal for a number of response mechanisms that cope with proteasomal inhibition, such as stabilization and prevention of aggregate formation by the induction of classical Hsps, elimination of polyubiquitinated proteins/aggregates by HDAC6 mediated aggresome formation and lysosomal uptake via autophagy, containment of inflammation by the induction of HO-1(heme oxygenase-1) and Nrf-2 (NF-E2-related factor 2) pathway and reduction of proteotoxic stress mediated apoptosis by the induction of classical Hsps and HO-1 [17–19] (Fig. 1B). Furthermore, nuclear translocation of NFκB (Nuclear factor κB) requires degradation of ubiquitinated phospho-IκB-α
undergo further reactions and finally form the age pigment lipofuscin which has toxic properties and accumulates in the lysosomal system. It can be assumed, as observed in skin aging. (F) When proteolytic capacity declines below a critical threshold of activity required to cope with oxidative stress, the final element into cellular metabolism, partly by inhibiting 20S proteasome. (E) Another side effect of proteasome inhibition is leading to a higher amount of phosphorylated physiological aging, the process of aggregation is slow[30,37,38]. The aggregate is independent from the original structure of the protein and introduces a new toxic for the degradation of oxidized proteins and less susceptible to direct oxidative stress, can be nevertheless diminished by aggregated oxidized proteins. These protein activation of activator protein-1 (AP-1), controlling MMP-1(matrix metalloproteinase-1) expression [22]. MMP-1 as a major protease of the extracellular matrix is thereby up-regulated causing increased extracellular protein degradation (Fig. 1E). Noteworthy, it can be expected that other AP-1 induced genes are also activated. This accumulation of aggregates is the final fate of protein damage under stress conditions. Under such circumstances oxidized proteins may not undergo appropriate proteolytic digestion but instead, cross-link with one another or form extensive hydrophobic bonds. It is believed that the cross-linked proteins react further with other cellular components, forming a fluorescent material referred to as lipofuscin (Fig. 1F). Lipofuscin is accepted to consist of oxidized proteins (30–70%) as well as lipids (20–50%) [23] and from the fifth decade of life, bound sugar residues were also detected in human lipofuscin [24].
hypothetical mechanism of lipofuscin formation was described in detail by Brunk and Terman in their widely accepted model known as “the mitochondrial–lysosomal axis theory of aging” [25]. According to this model, an intralysosomal accumulation of lipofuscin can be considered as the long-term result of a decreased degradation of oxidized proteins and an increase in intracellular free radical formation. Metals, including Fe, Cu, Zn, Al, Mn, and Ca, comprise up to 2% of lipofuscin [26] and especially intracellular free radical formation. Metals, including Fe, Cu, Zn, Al, Mn, and Ca, comprise up to 2% of lipofuscin [26] and especially catalytic iron seems to be an important factor in further oxidation reactions of the initial protein aggregate. In mammalian cells iron is the most abundant cellular transition metal and a fundamental player in the above mentioned mitochondrial–lysosomal axis theory of aging [25].

However, the detailed intracellular effects of lipofuscin in a cell are largely hypothetical and under discussion for a long time. By using an artificial lipofuscin it could be shown, that lipofuscin is a prominent source of oxidants and is able to incorporate iron in a redox-active manner (Fig. 2A). It could be shown that artificial lipofuscin and particularly iron-loaded artificial lipofuscin increase caspase-3 activity if taken up by cells and, therefore, apoptosis [27]. Furthermore, deferoxamine as chelating agent could reduce the effects of iron-loaded lipofuscin significantly. Thus, the amount of iron included in lipofuscin seems to play a crucial role in the intracellular effects of lipofuscin, particularly considering the ability to catalyze the formation of free radicals and the resulting cytotoxicity. These results match very well the hypothesis of Brunk and Terman [25], postulating that iron inclusions of lipofuscin result in a redox-active surface catalyzing the Fenton reaction.

Another major characteristic of lipofuscin is its ability to inhibit the degradation of oxidized proteins by competitively binding to proteolytic enzymes including the 20S proteasome as mentioned above as well as lysosomal proteases. How exactly the proteasomal substrate recognition functions is still under investigation, but one of the recognition motifs might be exposed hydrophobic amino acid structures on the surface of the highly oxidized and covalently cross-linked lipofuscin [5]. These structures seem to be main sites of proteasomal substrate recognition. After proteasomal binding to those structures the protease is unable to degrade the exposed structures completely because of sterical and/or mechanical inhibition by cross-links. So the proteasome is bound to the surface of lipofuscin in ineffective attempts of degradation detracting proteolytic capacity from other substrates and resulting in a measurable proteasomal inhibition. Furthermore, cellular viability is affected and significantly reduced [8]. Degradation of these exposed structures by using the effective and nonspecific protease K demonstrated that such unfolded peptide binding sites are largely responsible for the inhibition of the proteasome. This degradation of these binding sites significantly reduces the proteasomal binding and the inhibition of the proteasome could be partially prevented. Consequently less proteasomal capacity is detracted in futile attempts of degradation and cellular viability was unaffected or increased referred to the undegraded material (C) [29].

Fig. 2. Properties of lipofuscin. Studies have suggested that lipofuscin is not an inert waste product, but rather an active component influencing the cellular metabolism, which is especially relevant in senescent cells. It was proposed that lipofuscin is cytotoxic because of its ability to incorporate redox-active transition metals, resulting in a redox-active surface, able to catalyze the Fenton reaction: in the presence of Fe²⁺, H₂O₂ is decomposed forming hydroxyl radicals (OH•) [39]. The resulting Fe³⁺ can be reduced by superoxide and the vicious cycle starts again. This ability to incorporate transition metals (iron) and form oxidants was tested by using artificial lipofuscin-like oxidized, cross-linked protein aggregates (“artificial lipofuscin”). Non-iron-loaded artificial lipofuscin was incubated with Fe²⁺ and was able to incorporate a maximum amount of 7 mass%, matching the characteristics of native lipofuscin very well [26]. In vitro this material is able to generate the formation of free radicals and initiates apoptotic cell death, resulting in a significant loss of cellular viability (A). Another major and already demonstrated characteristic of protein aggregates/lipofuscin is the ability to inhibit the degradation of oxidized proteins by competitively binding to the proteasome [40–42]. The proposed mechanism for proteasomal inhibition is binding to exposed hydrophobic amino acid structures on the surface of the highly oxidized and covalently cross-linked lipofuscin [5]. These structures seem to be main sites of proteasomal substrate recognition. After proteasomal binding to those structures the protease is unable to degrade the exposed structures completely because of sterical and/or mechanical inhibition by cross-links. So the proteasome is bound to the surface of lipofuscin in ineffective attempts of degradation detracting proteolytic capacity from other substrates and resulting in a measurable proteasomal inhibition. Furthermore, cellular viability is affected and significantly reduced (B). Degradation of these exposed structures by using the effective and nonspecific protease K demonstrated that such unfolded peptide binding sites are largely responsible for the inhibition of the proteasome. This degradation of these binding sites significantly reduces the proteasomal binding and the inhibition of the proteasome could be partially prevented. Consequently less proteasomal capacity is detracted in futile attempts of degradation and cellular viability was unaffected or increased referred to the undegraded material (C) [29].
Aggregated proteins

Phagophore

Atg12-Atg5-conjugation system

Atg7 + Atg12

Atg7 + Atg12

Atg7 + Atg12

Atg7 + Atg12

Atg7 + Atg12

Atg12

Atg12

Atg12

Atg12

Atg12

Atg10

Atg10

Atg10

Atg10

Atg10

Atg10

Atg5

Atg5

Atg5

Atg5

Atg5

Atg16

Atg16

Atg16

Atg16

Atg16

Atg16

Atg16

Atg16

Atg16

Atg16

Atg16

LC3-II

LC3-II

LC3-II

LC3-II

LC3-II

LC3-II

LC3-II

LC3-II

LC3-II

LC3-II

LC3-II

LC3-II

LC3-II

LC3-II

LC3-II

LC3-II

LC3-II

LC3-II

PE

Fig. 3. The fate of aggregated proteins during functional macroautophagy. Macroautophagy is a process, which is responsible for the uptake of larger aggregates. Aggregates which are no longer degradable by the proteasome, but rather inhibit its proteolytic function are enclosed by a phagophore and transported towards the lysosomal system. Macroautophagy comprise the formation of an autophagosome, a double-membrane vesicle that engulfs substrates through the expansion of an isolation membrane, called phagophore. This isolation membrane is build, expanded and closed to an autophagosome and delivered to the endo/lysosomal system. Afterwards both structures fuse and the final structure is called autophagolysosome. The elongation of the phagophore requires two processes similar to ubiquitination: Atg12-Atg5-conjugation and LC3 (microtubule-associated-protein-light-chain-3)-modification [illustration of Atg12-Atg5-system and LC3 conjugation system according to Levine and Deretic [43]]. In the first conjugation system (Atg12-Atg5-system) Atg12 is conjugated with Atg5, in a process assisted by an E1-ubiquitin-like activating enzyme: Atg7 and the E2-ubiquitin-like conjugation enzyme: Atg10. Afterwards the Atg12-Atg5 conjugate forms a protein complex with Atg16 on the outside of the nascent autophagosome [44,45]. This complex is functionally necessary for the end product of the secondary conjugation system, lipidated LC3. First the cysteine protease Atg4 eliminates the C-terminal tail of LC3I to expose a glycine residue. A G116 is activated by Atg7, and LC3I is conjugated via a thioester bond with Atg7. Afterwards LC3I is transferred on the E2-ubiquitin-like conjugation enzyme Atg3, and again connected via a thioester bond. Finally LC3I is bound to the amino group of phosphatidylethanolamine (PE). This complex of LC3 with PE is called LC3II and localized via its lipid part to the autophagosomal membrane enabling membrane-elongation. Finally LC3II is deconjugated from the phospholipid-anchor via Atg4 and the luminal associated LC3II is degraded. This cycle of conjugation and deconjugation is important for the normal progression of autophagy. Especially under conditions of oxidative stress a huge amount of aggregated proteins accumulate. These aggregates are taken up by macroautophagy and react further with other cellular components, forming a fluorescent material referred to as lipofuscin, which accumulates in the lysosomal system over time.

Fig. 4. The fate of aggregated proteins during impaired or non-sufficient macroautophagy. Macroautophagy is maximally activated under stress conditions such as starvation, oxidative stress and conditions leading to enhanced protein misfolding [46–50]. This essential role of macroautophagy is underpinned by the fact that Atg5 or Atg7 null mice die a few hours after birth [51]. Nevertheless it is possible to investigate the role of macroautophagy in knockout models such as fibroblasts from mouse embryos deficient in Atg5 (Atg5−/− MEFs) under stress conditions. This ATG5−/− MEFS naturally show no Atg5 expression and thus no formation of the Atg12-Atg5-complex. As the Atg12-Atg5-conjugation system and LC3 modification [illustration according to Levine and Deretic [43]] are both mandatory for the formation of a phagophore, the uptake of aggregated proteins (and other substrates) is prevented in this knockdown system. The consequence is an increasing amount of cytosolic lipofuscin accumulation. These elevated levels of extralysosomal lipofuscin are combined with a decline in cellular viability and an increased amount of ROS production.
responsible for the uptake of larger aggregates. Normally aggregated proteins beyond proteasomal degradation are enclosed by a phagophore and mediated to the lysosomal system (Fig. 3). By application of oxidative stress to an ATG5 knockout model it was possible to manipulate the lipofuscin amount within lysosomes and the uptake of aggregates into lysosomes could be reduced. However this inhibition of macroautophagy actually did not prevent the formation of lipofuscin [32]. These findings indicate that in contrast to an earlier hypothesis [33,34] lipofuscin can also be formed in the cytosol (Fig. 4) and the uptake into an autophagosome seems to take place in a secondary step. Therefore, autophagosomes/lysosomes are not mandatory for the formation of lipofuscin but constitute a storage for aggregates reducing aggregate toxicity [32].

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