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HOW DO INTRACELLULAR PROTEOLYTIC SYSTEMS CHANGE WITH AGE?

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1. ABSTRACT

One of the common features of cells from senescent tissues is the accumulation of abnormal proteins. Several hypotheses have been proposed to explain the origin of those abnormal proteins. A defect in proteolytic systems usually responsible for the elimination of altered proteins from the cells could clearly contribute to such accumulation. Here we describe the effect of age on the major proteolytic systems within cells: the ubiquitin-proteasome pathway, the calcium-activated calpain pathways, and multiple lysosomal pathways.

Our group has contributed to the characterization of a selective pathway of degradation of cytosolic proteins in lysosomes that is activated under conditions of nutrient deprivation. In this lysosomal pathway of proteolysis proteins are transported through the lysosomal membrane assisted by cytosolic and lysosomal molecular chaperones and a receptor protein in the lysosomal membrane. The activity of this pathway significantly decreases with age, and this decrease might account for the cytosolic accumulation of aberrant substrate proteins in senescent cells. The cellular consequences of the decline of this lysosomal pathway together with possible methods to restore the reduced function are also addressed in this review.

2. INTRODUCTION: WHAT HAPPENS TO SENESCENT CELLS?

Aging is associated with a progressive reduction in almost all physiological functions. Senescent organisms show an impaired responsiveness to environmental stimuli and to physiological stress (1). A deterioration of the immune system in aging has also been described, and this immune system impairment is believed to contribute to increased mortality from infections, autoimmune diseases, and cancer in the elderly (reviewed in ref. 2).

At the cellular level, aging results in the inability of cells to proliferate. Senescent cells resemble cells that are arrested at the G1-S boundary of the cell cycle (3). A blockage in the expression of the c-fos gene, which leads to changes in the AP-1(active gene regulatory protein), along with changes in CREBP (cyclic AMP response element binding protein) and CTF (CAAAT transcription factor) transcription factor complexes, are believed to contribute to the loss of cellular proliferation in senescent cells (4, 5). Age-related changes in the activity of several cell cycle regulatory components such as Rb (retinoblastoma protein), Cdk2 (cyclin-dependent kinase 2), p21 (inhibitor of cyclin-dependent kinases), and p53 (tumor suppresser protein) seem also to participate in the mechanism of proliferation arrest in aging (reviewed in ref. 6). Genetic studies have shown that the senescent phenotype is dominant, and at least four separate genes are involved in the suppression of cellular proliferation (7). Senescent cells remain metabolically active for long periods of time, but there are progressive changes in cell structure and function. For example, cells accumulate somatic mutations in their DNA and lipofuscin-containing dense bodies in their cytoplasm (8).

Protein degradation together with protein synthesis take place continuously in all cells (9). Small modifications in the balance between these processes allow cells to rapidly adapt to changes in the extracellular environment (10). In addition to this continuous turnover of proteins inside cells, abnormally synthesized proteins or proteins incorrectly modified are also eliminated from the cells by the proteolytic systems (11). Intracellular proteases also participate in many other fundamental cell processes including cell differentiation, cell cycle progression, antigen presentation, and intracellular traffic of proteins (reviewed in ref. 12).

Because of these many cellular functions in which the proteolytic systems participate, the consequences of a reduction in intracellular protein degradation are widespread. Along with a severe difficulty in adapting to environmental changes, cells with impaired protein degradation are less able to eliminate abnormal or damaged proteins. Accumulation of those altered proteins may cause a variety of further problems within cells. In addition, defects in the processes of intercellular communication (i.e. antigen presentation, hormone and peptide secretion) will make those cells more vulnerable to the attack of exogenous agents and unable to develop a coordinated systemic response. These changes may contribute to common phenotypes of senescent cells, including the inability to progress through the cell cycle (reviewed in ref. 13).

Several hypotheses have been proposed about the mechanisms responsible for aging. A detailed description of each of these theories is beyond the scope of this review (for review see ref. 13). However, in general, these theories can be separated into catastrophic (aging is the result of damage accumulation throughout the cells' lifespan) and genetic (aging is due to a genetic clock that causes age-related phenotypes in cells). Lately, a multifaceted origin for senescence is becoming generally accepted (14-16). Thus, along with a genetic program, the effect of extracellular agents during the cellular lifespan modulates the severity of the age-related alterations. It is in that modulation that proteolytic systems play an important role. In many different tissues including liver, cardiac and skeletal muscle, and brain, a decline in protein degradation with age has been described (17-22).

Several proteolytic systems participate in intracellular protein degradation (reviewed in ref. 23). They mainly differ in their intracellular localization and in the regulation of their activities. We will consider the ubiquitin-proteasome proteolytic pathway located in the cytosol and nucleus, the calpains located in cytosol and associated with the cytoskeleton, and several different pathways of lysosomal proteolysis. Although all of these degradation pathways are present in all cells, the activity of each of these systems varies from tissue to tissue and also depends on environmental conditions. Specific groups of substrate proteins have been assigned to each of these proteolytic pathways, but it is becoming apparent that some proteins can be substrates for multiple proteolytic systems. Thus, single proteins have been described to follow one or another proteolytic pathway depending on the cellular conditions (i.e. c-fos and c-jun (24); IkB (25); membrane receptors (26), and components of one proteolytic system influence the activities of other proteolytic systems (27-30). In the present work, we have first addressed what is known about the effect of age on the main intracellular proteolytic systems, followed by several examples of how those systems are differently affected by age depending on the tissue analyzed.

3. MODEL SYSTEMS FOR THE STUDY OF AGING

There are many different model systems used for the study of aging. For some studies, it is possible to work with whole animals of different ages, and genetically defined strains of mice and rats are commonly utilized (31, 32; reviewed in ref. 33). A mouse model of accelerated senescence has also been established (34), and senescence biomarkers on those animals have been very well characterized (35-37; for review see ref. 38). Recently, transgenic mice have also proved to be useful in the analysis of spontaneous mutations accumulating during aging (39). In addition, the insertional mutation of a gene in one of those transgenic mice has allowed the identification of a new gene, *klotho*, that is involved in the suppression of several aging phenotypes (40).

In order to simplify the study of aging in organisms, animal species with shorter lifespans than mammals have also been used. Thus, the nematode *Caenorhabditis elegans*, with a lifespan of 20 days and 1% of the mammalian genome, has allowed an extensive analysis of the genetics of senescence (reviewed in ref. 41). Some insects, such as *Drosophila melanogaster*, with a relatively short lifespan (1-3 months) but with more genetically heterogeneous populations, have been utilized for the analysis of genetic influences on lifespan (41).

Some unicellular organisms, such as the yeast *Saccharomyces cerevisiae,* have considerably simplified studies in aging. The highly developed molecular biology of yeast and the ability to select for mutants in this microorganism have allowed the identification of several genes that show altered expression with age. Still, the senescence of budding yeast is a process that differs in several respects from the senescence in multicellular organisms (41).

The study of some cellular processes is difficult to perform in whole aged animals, since compensatory mechanisms are frequently activated. Several *in vitro* models of aging have also been established (reviewed in ref. 42). Specific cell types such as human diploid fibroblasts, endothelial cells, keratinocytes and lymphocytes have been shown to have finite replicative lifespans in culture. DNA synthesis and cell division cease, but metabolic activity and cellular viability may be maintained for an extended period of time (43). The loss of proliferation in these *in vitro* systems is interpreted as an expression of aging at the cellular level. The more relevant facts of those senescent cultures and their correlations with

Table 1. Senescent fibroblasts as a model of aging

CHARACTERISTICS OF SENESCENT CELLS IN CULTURE	REFERENCE
❑ The proliferative potential of cells depends on the number of cell doublings undergone and is independent of the chronological age of the cells	44
❑ There is an inverse relationship between the age of a donor and the proliferative potential of their fibroblasts *in vitro*	45
❑ There is a correlation between the proliferative potential of cells from different species and the average life span of that species	46
❑ Cells from patients with premature aging disorders show reduced proliferative potential	47
❑ Senescent fibroblasts in culture display enlarged, flattened morphology and fail to replicate their DNA in response to normal growth stimuli	48

aging in complete organisms are summarized in table 1. Whether or not aged organisms contain cells that have reached the end of their proliferative potential has been a controversial topic. However, the analysis of variant beta-galactosidase activities has recently been successfully applied to identify senescent cells in aging skin *in vivo* (49).

4. INTRACELLULAR PROTEOLYTIC SYSTEMS AND AGING

Senescent human fibroblasts in culture have been very helpful in identifying the defective proteolytic systems with age. General techniques for the study of intracellular protein degradation in cultured cells, such as metabolic radiolabeling of intracellular proteins or microinjection of radiolabeled proteins, and further analysis of their intracellular breakdown have demonstrated that total protein degradation was considerably slowed in old fibroblasts when compared with young cells (19, 50-53). The addition of specific protease inhibitors to the culture medium and/or the analysis of protein breakdown under specific cellular conditions has been used to implicate the protease or proteases responsible for the decrease in protein degradation rates with age.

In recent years, several cell-free models have been developed to analyze independently the function of some of the major proteolytic systems of cells. Thus, ubiquitination of proteins can be accomplished *in vitro* with rabbit reticulocyte or *Xenopus* egg extracts (54, 55), the 20S proteasome has been isolated from several different organisms and its proteolytic activities carefully characterized (56, 57), endosomal/ lysosomal fusion can be reproduced with isolated vesicles in vitro (58), and direct transport of proteins through the lysosomal membrane can be followed in isolated lysosomes (59-61).

4.1. Cytosolic proteolytic systems: Proteasomes and calpains

4.1.1. Ubiquitin-proteasome system

Several proteases have been described in the cellular cytosol. The 20S proteasome, a multicatalytic protease complex, is without doubt one of the most studied and best characterized cytosolic proteases. The 20S proteasome is normally found associated with several heterogeneous proteins that form one or two 19S caps at the ends of the 20S structure, that regulate its proteolytic activity (62). This complex is called the 26S proteasome (reviewed in ref. 63). The nature of many of these regulatory subunits remains unclear, but ATPase, ubiquitin-binding, and ubiquitin isopeptidase activities have been detected (64).

The 26S proteasome has been considered mainly responsible for the degradation of short-lived and abnormal proteins in the cytosol and nucleus (reviewed in ref. 65). However, recent evidence for the participation of this proteolytic system in basal protein turnover of cytosolic proteins has also been presented (66). In addition, not only cytosolic proteins, but also previously compartmentalized proteins, can be redirected to the cytosol where they are degraded by the 26S proteasome (67). Degradation of proteins by the proteasome usually requires a previous covalent modification of the substrate protein in order to be recognized by the proteolytic complex. This modification, known as ubiquitination, consists of the covalent attachment of a 8.6 kDa protein (ubiquitin) to the epsilon-amino group of a lysine residue of the substrate protein (reviewed in ref. 68). Covalent attachment of ubiquitin to the protein substrate is mediated by a complex group of enzymes (activating enzyme (E1), carrier/conjugating enzymes (E2) and conjugating enzymes (E3)) (see figure 1) (69). Ubiquitin can also be covalently attached to itself to form a ubiquitin chain on a substrate protein that is then targeted for degradation by the 26S proteasome (70). Degradation of polyubiquitinated proteins seems to be facilitated by the direct interaction of some subunits of the proteasome with the multiubiquitin chain (71, 72). Other enzymes are able to remove ubiquitin from proteins (deubiquitination), allowing it to recycle (73, 74). Ubiquitination is required for degradation of many important regulatory proteins such as cyclins, c-fos, and p53 (68).

Although ubiquitination is the most generalized target signal for protein degradation by the 26S proteasome, ubiquitin-independent degradation of some proteins by the proteasome has also been reported (i.e. ornithine decarboxylase) (75). On the other hand, monoubiquitination of some membrane proteins in their cytosol-exposed region targets them for degradation by the vacuolar (lysosomal) system in yeast (76, 77).

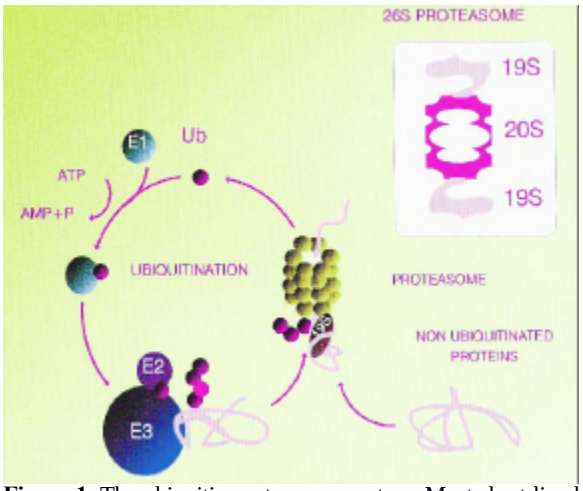

Figure 1. The ubiquitin-proteasome system. Most short-lived and abnormal proteins in the cell cytosol are degraded by the 26S proteasome, directly or after a covalent modification known as ubiquitination. The multiple enzymatic steps and several enzymes required for the ubiquitination of proteins are summarized in this figure. See the text for details.

Regarding the proteolytic core of the proteasome, at least three different neutral peptidase activities have been described (78). Recently, specific inhibitors for this multicatalytic complex have been developed (79-81). The physiological activators described for the 20S proteasome are located at the 19S ATPase complex. In addition, in the presence of detergent, fatty acids, or polylysine, the activity of the proteasome significantly increases (82), but the physiological significance of such regulation is not known. The proteasome normally exists in a latent state in the cell (65) which may be important in preventing uncontrolled proteolysis (78). Lately, several authors have demonstrated that the subunit composition of the proteasome changes depending on specific stimuli, and that these altered subunits result in changes in the various proteolytic activities (83).

A decrease in a non-acidic protease activity with age has been reported in several types of cells (84, 85). The 26S proteasome is one possible candidate for that proteolytic activity. However, no clear evidence for such age-related changes in proteasome activity has been found. A detailed analysis of each of the peptidase and proteolytic functions of the 20S proteasome along with its intracellular levels in senescent cells has been performed by Shibatani and Ward (86). These authors, working with rat cytosolic extracts, found no differences with age in the intracellular levels or the total proteolytic activity of the 20S proteasome (tested with casein, a well established substrate). Only after SDS stimulation was an increase in the chymotrypsin and trypsin-like activities found in the aged hepatocytes. Under those conditions the peptidylglutamyl peptide-hydrolyzing activity of the 20S proteasome was clearly decreased, but that has been shown to be of minor importance compared with the other proteasome activities, at least in yeast (87, 88). Therefore, Shibatini and Ward (86) concluded that the reported changes in 20S proteasome activity with age are not sufficient to account for the decreased protein degradation in old cells.

Similar results were obtained by Sahakian *et al*. (89) working with purified rat liver 20S proteasome instead of cellular extracts. In addition, the decrease in non-acidic protease activity with age, first described in rat heart and liver, seems to be restricted to some organisms and tissues since recent studies have shown that this activity is not modified in houseflies or in rat brain with age (90).

In the absence of changes in the proteasome activity, a reduction in protein ubiquitination could also result in reduced intracellular protein breakdown. Several changes in intracellular protein ubiquitination with age have been reported. Studies in senescent human fibroblasts revealed that aged cells have less free ubiquitin and more ubiquitin-protein conjugates than young cells (91). Similar changes have also been observed in lens (92) and in brain (93). However, levels of ubiquitin mRNA do not change with age, and there is no difference in the ability of senescent fibroblasts to degrade ubiquitinated proteins (91). The fact that changes in protein ubiquitination do not always indicate changes in protein degradation rates may reflect the participation of ubiquitin in intracellular processes other than protein degradation (94-96). Although these results indicate that a reduction in the ubiquitin-proteasome pathway is not a common finding in aging, this proteolytic pathway may decline in certain tissues under particular conditions. For example, as described in detail below, under oxidizing conditions, a failure in the ubiquitination process in senescent eye lenses seems to contribute to the accumulation of abnormal proteins (97).

4.1.2. Calpains

Calpains, or calcium-dependent proteases, constitute the other major cytosolic proteolytic system (reviewed in ref. 98). The activity of these neutral, thiol proteases is tightly regulated by intracellular calcium levels. Micromolar calcium concentrations activate micro-calpain, but millimolar calcium concentrations are required for milli-calpain activation (99). Where in the cell millimolar calcium concentrations may be achieved remains an unsolved problem. Translocation of calpains to the cell membrane and their limited autolysis once there result in calpain activation (100, 101) (figure 2). Calpains partially degrade membrane and cytoskeletal proteins and several membrane-associated enzymes (102-104). The limited proteolysis of certain membrane proteins by calpains has been shown to be necessary for the process of membrane fusion (105). Two of the best characterized substrates for the calpain system are the band 3 protein (an anion exchanger) in the erythrocyte membrane (99) and protein kinase C (106).

An increase in proteolysis of band 3 protein by calpains in erythrocytes of old individuals has been described (107). This increased degradation of band 3

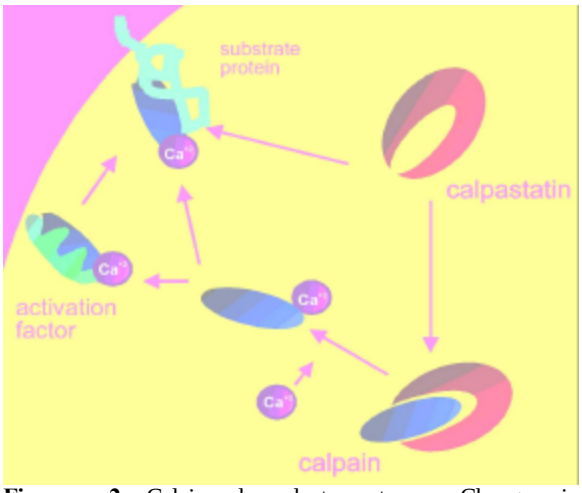

Figure 2. Calcium-dependent proteases. Changes in intracellular calcium levels modulate the activity of two cytosolic thiol proteases (calpains). Those proteases are usually present in an inactive form associated with calpastatin, an intracellular inhibitor. After translocation to the cell membrane and limited autolysis, calpains are activated. Calpains are responsible for the degradation of certain membrane and cytoskeletal proteins. See the text for details.

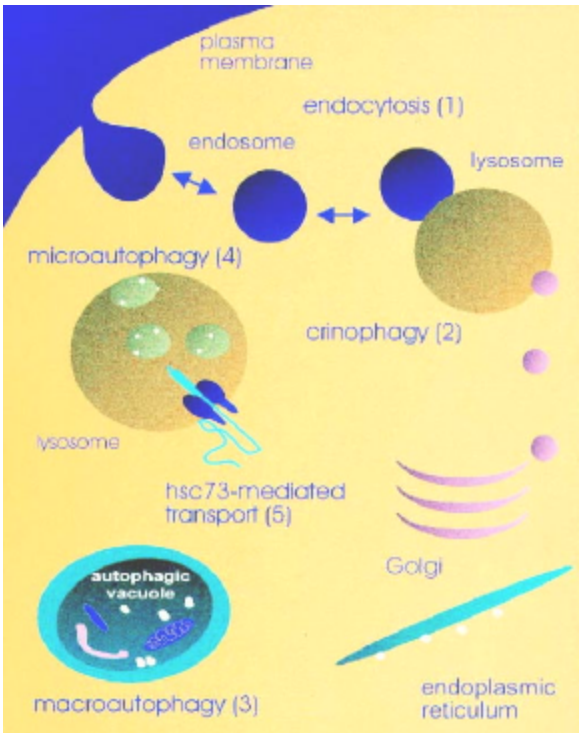

Fig. 3. Lysosomal pathways of protein degradation. Intra- and extracellular proteins can reach the lysosomal matrix by different mechanisms: vesicular fusion (endocytosis (1) and crinophagy (2)), macroautophagy (3), microautophagy (4), and direct transport through the lysosomal membrane (5). See the text for details.

protein correlates with a higher translocation of calpains from the cytosol to the cell membrane. A diminution of thiols in senescent cells, as a consequence of free radical reactions and oxidative damage (108), may cause enhanced calpain translocation to the cell membrane (100). The increase in degradation of band 3, and probably other membrane proteins, modifies the stability of the erythrocyte membrane and reduces erythrocyte life span in aged animals and humans (109). It is likely that this increase in degradation of membrane proteins described in erythrocytes is also common for most cell types. For example, as described below, the abnormal proteolysis and neuronal degeneration in Alzheimer's disease may be, in part, a consequence of calpain activation (110).

The age-related changes in the calpain system seem not to be related with changes in the proteolytic activity of calpains themselves, because when calpains from young individuals were added to membranes from old erythrocytes an increase in membrane protein degradation was also observed (109). Those authors suggest the existence of specific changes in cellular membrane proteins with age which result in an increased affinity of those membranes for calpains thereby bringing the protease close to its potential substrate proteins. Age-related changes in the substrate susceptibility to proteases should also be considered. Several protein modifications make proteins more susceptible to proteolytic attack. Those susceptibility changes have been extensively studied in the brain, and we will review them in more detail in the section of protein degradation in the central nervous system. Finally, possible modifications in the activity of calpastatin, the physiological inhibitor of calpains, with age need also to be considered (101).

4.2. Lysosomal function in senescent cells

Lysosomes are organelles that contain a powerful mixture of proteases, peptidases, and other hydrolases capable of degrading most intracellular and extracellular macromolecules (reviewed in ref. 111). The proteases responsible for the degradation of proteins inside lysosomes are called cathepsins (reviewed in ref. 112). In general, lysosomes have been considered to be responsible for the continuous basal turnover of most intracellular proteins (mainly long-lived proteins) in liver, kidney and certain other tissues.

Several types of evidence have identified lysosomes as one of the major proteolytic systems affected by age: (i) predominantly, degradation of long-lived proteins is retarded in senescent fibroblasts (13, 53) and in livers of aged rats (113); (ii) in most cells there is an age-related increase in number and size of lysosomes (114); (iii) storage bodies similar to those described in senescent cells become evident in young cells after inhibiting lysosomal proteolysis with cathepsin inhibitors (115).

When considering the age-related reduction in lysosomal function, it is necessary to separately analyze the different mechanisms by which proteins can be transported into lysosomes. The best characterized of these mechanisms are: vesicular fusion (endocytosis and crinophagy), macroautophagy, microautophagy and the direct protein transport through the lysosomal membrane (reviewed in ref. 23) (figure 3).

4.2.1. Fusion of lysosomes with vesicular compartments

Primary lysosomes are able to completely degrade the protein content of several kinds of intracellular vesicles after membrane fusion. Vesicles containing secretory proteins originate at the Golgi apparatus, and they normally release their protein content to the extracellular medium after fusion of the vesicles with the plasma membrane. Under specific conditions, when a decrease in protein secretion is required, 20-70% of those vesicles directly fuse with lysosomes in a process called crinophagy, after which secretory proteins are completely degraded (116, 117). In spite of the several methods developed to analyze this vesicular traffic (reviewed in ref. 118), the intrinsic mechanisms regulating this fusion process are still unclear.

Fusion of lysosomes with endosomes, vesicles containing several extracellular and plasma membrane proteins, is fairly well-characterized (for review see ref. 119). Internalization of several membrane receptors after ligand binding takes place by receptor-mediated endocytosis into clathrin-coated vesicles and subsequent traffic of those vesicles through the endosomal/lysosomal pathway. In that process membrane receptors may be recycled to the cell surface while the ligand is completely degraded after fusion of late endosomes with lysosomes. In other cases the receptor is not recycled and is delivered to lysosomes for degradation along with the ligand. Endocytosis is also required for uptake of extracellular nutrients, maintenance of cell polarity, and presentation of antigenic peptides. Extensive reviews of the movement of membrane receptors through different intracellular compartments and the signals that trigger receptor internalization and receptor recycling have recently been published (119, 120).

A decrease in levels of some serum proteins (121) and plasma membrane proteins (122) with age has been described. Those preliminary reports suggested some alterations in the endocytic system responsible for the internalization and degradation of those proteins. However, more direct studies of the endocytic system in senescent cells suggest little change in this process with age. An increase in the internalization of latex beads in senescent fibroblasts has been found (123), and a decrease in the endocytic activity of aged fibroblasts and Kupffer cells has also been reported (124, 125). In contrast, internalization and degradation of low density lipoprotein and epidermal growth factor are unmodified in senescent fibroblasts (126, 127). These discrepant results may be related to the different analyses of the experimental data performed or in cell type analyzed in each study. Thus, Gurley and Dice (128) showed that endocytosis values should be expressed per microgram of cell protein instead of normalized per number of cultured cells, since old cells are much larger than young cells. Under those circumstances there was no difference in endocytosis rates for young and old fibroblasts. In addition, rates of fluid-phase and absorptive endocytosis were not modified with age. Thus, there is not a general failure in lysosomal function during senescence.

4.2.2. Macroautophagy and microautophagy

A large number of intracellular proteins can be degraded in lysosomes by a process called macroautophagy (reviewed in ref. 129). In this process, complete regions of the cytoplasm, including cytosolic proteins as well as entire organelles, are surrounded by a membrane to become double-membraned structures known as autophagosomes or autophagic vacuoles (figure 3). The proteins inside autophagosomes are then completely degraded after fusion with lysosomes. In organs such as the liver, macroautophagy accounts for most intracellular protein degradation, especially during early starvation (130). Factors inhibiting this process include amino acids, insulin, cell swelling, and disruption of the cytoskeleton (131, 132). Among the mechanisms controlling macroautophagy, protein phosphorylation plays an important role (133). Although proteins in the sequestered cytoplasm appear to be taken up nonselectively, under certain conditions selectivity in this degradative process has been demonstrated. For example, peroxisomal autophagy can be preferentially activated under conditions where peroxisomes are no longer needed (134, 135). In addition, selective degradation of specific cytosolic proteins in the yeast vacuole following a novel vesicular pathway has been recently described (136). The fusion of lysosomes with the autophagosome follows similar mechanisms as those previously described for the endocytic and secretory pathways, and evidence of interaction between both processes has been presented (137).

During senescence, severe changes in the autophagosome/lysosomal system have been described. Degradation of several proteins microinjected into the cytoplasm of senescent human fibroblasts is significantly lower than in young fibroblasts (20). For some of those proteins (RNase S protein, lysozyme), their degradation is believed to follow the autophagosome/lysosomal pathway. The half-lives of some of those microinjected proteins are summarized in table 2. A decrease in autophagosome formation as well as a decrease in the degradative activity of lysosomes would result in the slower degradation of substrate proteins. Since autophagic vacuole formation rates decrease in senescent cells (138), and rates of elimination of autophagic vacuoles also decrease with age (139), reductions in autophagosome formation and fusion with lysosomes both contribute to the age-related decline in macroautophagy.

A second process of intracellular protein sequestration by lysosomes has been called microautophagy. During microautophagy only small portions of cytoplasm are engulfed by small invaginations in the surface of lysosomes. After internalization of those vesicles and breakage of their surrounding membrane, the cytoplasmic proteins are degraded inside lysosomes (140). In contrast with macroautophagy that is mainly activated under conditions of nutrient deprivation, microautophagy is responsible for the continuous basal degradation of long-

Table 2. Age-related changes in the half-lives of several cytosolic proteins

	$T_{½}$ (h)			
Protein	Young	Old	Model System	Reference
Aspartate aminotransferase	55	90	Human fibroblast	152
Lysozyme	28	80	Human fibroblast	20
Aldolase	175	520	Human fibroblast	20
RNase S-protein	88	120	Human fibroblast	20
RNase A	100	185	Human fibroblast	20
Poly-Glu:Tyr:Ala	110	375	Human fibroblast	20
Ornithine decarboxylase	0.15	0.5	Mouse Liver	152
Aldolase	26	37	Mouse Liver	152
Aldolase	35	170	Nematode	152
Enolase	58	161	Nematode	152
Triosephosphate isomerase	64	120	Nematode	152
Ovalbumin	106	164	Rat hepatocyte	195
Horseradish peroxidase	50	75	Rat hepatocyte	195

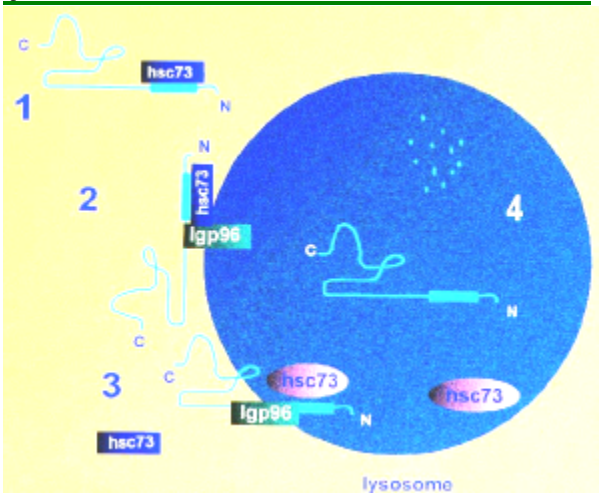

Figure 4. The hsc73-mediated pathway of cytosolic protein degradation. The main components of this system of direct transport of proteins through the lysosomal membrane are: a cytosolic chaperone of 73 kDa (hsc73), a receptor protein at the lysosomal membrane (lgp96) and an intralysosomal form of the cytosolic chaperone (lys-hsc73). The substrate protein after interaction with hsc73 (1) is directed to lysosomes where after binding (2) and transport through the lysosomal membrane (3), it is completely degraded in the lysosomal matrix (4).

lived proteins (141). No information is available concerning rates of microautophagy in aging.

4.2.3. Direct transport of cytosolic proteins through the lysosomal membrane.

A third mechanism of lysosomal degradation of certain cytosolic proteins occurs after direct transport through the lysosomal membrane (reviewed in ref. 23). This protein transport resembles in many aspects the transport of proteins into other cellular compartments (142). At least two molecular chaperones and a receptor protein, elements also described for other transport systems, are also present in this lysosomal pathway. The sequence of events that directs specific cytosolic lysosomes to their lysosomal degradation by this pathway is summarized in figure 4. The substrate proteins contain in their sequence a consensus peptide motif biochemically related to the pentapeptide, KFERQ (143, 144), that is recognized by a cytosolic chaperone of 73 kDa (hsc73) (143, 145). The binding of hsc73 to that region of the substrate protein directs the complex toward the lysosomal membrane (59). A lysosomal membrane glycoprotein of 96 kDa (lgp96 in rats or lamp2 in humans and mice) acts as a receptor for the substrate protein (146). Assisted by a form of the cytosolic hsc73 in the lysosomal matrix (147, 148), the substrate protein reaches the lysosomal matrix where it is completely degraded. Binding to the lysosomal membrane and uptake into the lysosomal matrix are the rate-limiting steps in this process and are directly related with lysosomal levels of lgp96 and hsc73, respectively (146, 148).

This hsc73-mediated lysosomal pathway was first described in human fibroblasts in culture (51, 149, 150) and then characterized in rat liver (60, 151). The direct transport of proteins into lysosomes is activated by serum deprivation of confluent cultured cells (152) or prolonged starvation of intact animals (153, 61). Approximately 30% of the cytosolic proteins are degraded by this pathway (153). Several of the substrates already identified for this pathway are: ribonuclease A (150), glyceraldehyde-3-phosphate dehydrogenase (60), aldolase (60), some subunits of the 20S proteasome (154), transcription factors such as c-fos (155) and IkB (Cuervo, A.M., Hing, M., Lim, B. and Dice, J.F., unpublished results), and alpha-2-microglobulin, a secretory protein that is also located in the cytosol of hepatocytes and kidney proximal tubular cells (Cuervo, A.M., Hildebrand, H., Bomhard, H. and Dice, J.F., unpublished results).

Analysis of the degradation of proteins microinjected into senescent fibroblasts revealed a decrease in the degradation of ribonuclease A, one of the described substrates for the direct lysosomal pathway (152) (figure 5A). In addition, differences between young and old cells in total protein degradation rates were especially evident in confluent cells after serum withdrawal, a condition under which the hsc73-mediated transport of proteins into lysosomes is activated.

Working with lysosomes isolated from old rats we have also observed a decrease in their ability to directly take up substrate proteins for this pathway (Cuervo and Dice, unpublished results) (figure 5B). All those results suggest that the direct transport of cytosolic proteins through the lysosomal membrane is impaired in old cells, and this reduction is at least partially responsible for the age-related decrease in total protein degradation. The components of the hsc73-mediated pathway that are affected by age are currently being studied in our laboratory.

Table 3. Changes in several protein degradation pathways in specific cell types with age

			LYSOSOMAL		
Tissue	**Ubiquitin/ Proteasome**	**Calpains**	**Macroautophagy**	**Microautophagy**	**Hsc73-mediated**
Central nervous system		Increase			
	No change	Decrease	No data	No data	No data
Skeletal muscle	No change	No change	No data	No data	No change
Liver	No change	No change	Decrease	No data	Decrease
Eye lens	Decrease	No change	No data	No data	Decrease

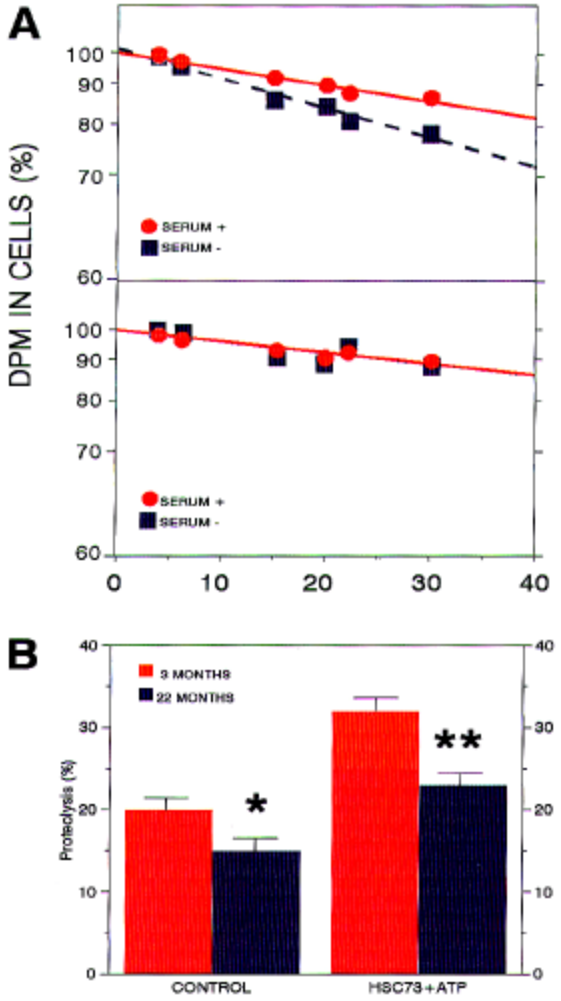

Figure 5. Evidence for reduction of the hsc73-mediated pathway with age. Decrease in the degradation of microinjected RNase A in old fibroblast when compared with young fibroblasts, especially after serum deprivation (**A**), and a reduced ability for the direct uptake of this protein by isolated liver lysosomes from old rats (**B**) strongly support a reduction of the hsc73-mediated pathway of protein degradation with age.

In conclusion, the analysis of the effects of senescence on the lysosomal function reveals that, rather than a generalized failure in the lysosomal system with age, specific degradative mechanisms are impaired in senescent cells. The important role of lysosomes in bulk protein turnover in liver, kidney, brain and fibroblasts make them mainly responsible for the age-related decrease in protein degradation in those organs.

5. TISSUE-SPECIFIC CHANGES IN PROTEOLYTIC SYSTEMS WITH AGE

We discuss here four representative examples of changes in protein turnover that are organ-dependent. The age-related modifications in the activity of several protein degradation pathways in those tissues are summarized in table 3.

5.1. Central nervous system

The central nervous system is one of the tissues that shows the most dramatic changes in protein degradation with age. Age-related morphological and neurochemical changes in brain cells have been very well-characterized, and many of them can be reproduced after exposure to different protease inhibitors (156, 157). Consequently, reduced protease activities or enhanced protease inhibitor activities might be involved in brain aging.

Several age-related changes in the activity of brain proteases have been reported (see table 4). In addition to changes in levels and activity of brain proteases with age, alterations in their substrate sensitivity to proteolytic attack need to be considered. For example, there are many reports of increased levels and activities of cathepsin D and calpain in the aged brain, but such changes do not correlate with an increased protein degradation, since proteolysis declines with age (169). In the same way, the accumulation of lipofuscin-pigment in lysosomes is not due to a decrease in cathepsin B activity with age (170). These discrepancies and others summarized in table 4 may be due to the different substrate proteins used (reviewed in ref. 169).

The lysosomal/endosomal and calpain systems seem to be the most affected by age in neuronal tissues. Thus, as described above, lysosomes undergo marked morphological changes in senescent cells and participate in neurodegenerative processes. As a result of aging or metabolic and oxidative stress, indigestible or incompletely degraded material accumulates within the endosomal-lysosomal system and produces a heterogeneous group of residual bodies containing lipofuscin (171-173). Although those aggregates can also be caused by treatment with lysosomotropic inhibitors (174), their accumulation in

Table 4. Age-related changes in protein degradation in the nervous system

CHANGES IN PROTEASE ACTIVITY	REFERENCE
❑ Increase of calpain activity in spinal cord	158
❑ Increase in calpastatin levels in brain	159
❑ Increase in calpain and cathepsin D activity in brain	160
❑ Decrease in calpain levels but increase in calpain activity in Alzheimer's disease patients	110,161
❑ Increase in levels of cathepsin D in brain	162
❑ Increase of brain calpain activity depends on the cerebral area analyzed	163
❑ Increase in cathepsin D, E, and B activities and decrease in cathepsin L activity in brain	162
CHANGES IN SUBSTRATE SUSCEPTIBILITY TO PROTEASES	
❑ Microtubule-associated proteins in brain are more susceptible to cathepsin D-like proteases	164
❑ Phosphorylation and carbonyl modification of neurofilament proteins reduce their proteolytic susceptibility	161, 165, 166
❑ Age-related modifications of synaptosomal membrane proteins inhibits their proteolysis	167
❑ Binding of aluminun to neurofilament proteins inhibits their proteolysis	161
❑ Peroxidation of myelin membrane proteins increases their susceptibility to proteolysis	168

aging does not appear to be due to reduced protease activity but to changes in proteolytic susceptibility of the substrate proteins. An increased expression of some lysosomal proteases (cathepsin D, B and E) (175) together with a decreased stability of the lysosomal membrane (176) have also been described in senescent brain.

In Alzheimer's disease, one of the most common neurodegenerative diseases, proteins of the cell membrane and cytoskeleton are abnormally processed and accumulate in the brain. It is now believed that lysosomal alterations are important in the pathogenesis of this disease and in the accumulation of amyloidogenic peptides (177-179). Acid hydrolases accumulate in atrophic and degenerating neurons, and their release to the extracellular space may contribute to senile plaque formation (179). The typical amyloid deposits in Alzheimer's disease are normally produced in an acidic compartment by noncysteine proteases, and then they are eliminated by lysosomal cysteine proteases (mainly cathepsins B and L) (177, 180-182). Treatment with general cysteine proteinase inhibitors cause accumulation of potentially amyloidogenic precursor protein fragments (183, 184).

5.2. Liver and cultured fibroblasts

In spite of the many differences between hepatocytes and fibroblasts, the parallelism of most of the results regarding age-related changes in intracellular protein degradation lead us to consider them together in this section. Many of the age-related changes in the degradative systems described in section 4.2. were obtained using rat liver or human fibroblasts in culture, and these results are summarized in table 5.

The liver is one of the organs in which a decrease in total protein degradation with age has been well-established (113). The most affected proteolytic system in liver from old animals is the lysosomal system. Lysosomes are responsible for more than 80 % of protein degradation in liver (196, 197). Characteristic age-related changes in the morphology and function of the macroautophagy/lysosomal system have been described in hepatic cells: there is an increase in number and size of lysosomes in liver cells with age (192); the activity of lysosomal enzymes increases with age in parenchymal and non-parenchymal cells of the liver (193); there is a decrease in the formation of autophagic vacuoles and in the degradation of their content (141); the half-lives of proteins microinjected in hepatic cells increase with the age of the donor animal (table 2; 195); and the selective uptake of cytosolic proteins by lysosomes from rat liver decreases with age (figure 5B; Cuervo and Dice, unpublished results). This decrease in the activity of the hsc73-mediated lysosomal pathway in rat liver with age is considered to be one of the major proteolytic pathways responsible for the decrease in total protein breakdown in liver.

As described previously, human fibroblasts in culture have been extensively used for studies of aging (see section 3). In spite of the controversy in accepting senescence in culture as a good model of aging *in vivo*, fibroblasts in culture show very similar alterations in protein degradation pathways as does liver from aged rats. table 5 summarizes these changes in proteolytic pathways.

5.3. Skeletal muscle

In contrast to the nervous system, liver, and fibroblasts, where lysosomes appear to play a key role in alterations of protein degradation associated with aging, the ubiquitin-dependent system is mainly responsible for protein degradation in skeletal muscle, especially in many muscle-wasting conditions (198, 199). During aging there is a progressive loss of muscle mass (200) that originates because of an imbalance between synthesis and degradation of proteins (reviewed in ref. 201). However, when basal rates of skeletal muscle protein turnover are analyzed, there is a significant reduction with age in both synthesis and degradation. Studies analyzing rates of protein synthesis by [^{14}C]tyrosine incorporation into muscle, and degradation by urinary excretion of 3-methylhistidine, related to myofibrillar protein breakdown, have demonstrated that both processes significantly decrease in skeletal muscle of rats with age (202). The protein synthesis/degradation imbalance in senescence is only evident after induction of a catabolic state such as in starvation, denervation atrophy,

Table 5. Comparison of the age-related changes in protein degradation in rat liver and cultured human fibroblasts

PROTEIN DEGRADATION	LIVER		FIBROBLASTS	
	CHANGE	REFERENCE	CHANGE	REFERENCE
❑ Total protein degradation	Decrease	113	Decrease	185
❑ Proteolytic susceptibility	Increase	186	Increase	187
❑ Oxidized and modified proteins	Increase	188	Increase	189
PROTEOLYTIC SYSTEMS				
Cytosolic				
❑ Proteasome				
• Proteasome levels	No change	86	No change	190
• Degradation of ubiquitinated proteins	No change	191	No change	91
❑ Calpain activity	No data		Increase	110
Lysosomal				
❑ Elimination of autophagic vacuoles	Decrease	141	Decrease	140
❑ Half-life of microinjected proteins	Increase	195	Increase	20
❑ Hsc73-mediated	Decrease	Cuervo & Dice, unpublished	Decrease	20
❑ Number and sizes of lysosomes	Increase	192	Increase	114
❑ Activity of cathepsins	Increase	193	Increase	194

cancer, acidosis or trauma, and reflect a failure to rapidly restore muscle protein after those circumstances (203). Those authors also demonstrated that muscle wasting resulted from increased proteolysis in normal rats, but it is caused by a decrease in protein synthesis in senescent rats. Under catabolic conditions only a small increase of cathepsin D and milli-calpain have been observed, but it does not modify total protein breakdown (204).

5.4. Eye lens

Another tissue where age-related changes in the activity of proteolytic systems has been studied is the eye lens. In the lens the proteolytic removal of damaged proteins may play an important role in maintaining the lens transparency (90, 205-207). Normal lenses have the ability to increase ubiquitin conjugation activity in response to oxidative stress, but that response is impaired in senescent lenses (92, 97). No changes in levels of ubiquitin conjugating enzymes in lenses were detected with age, but changes in their ability to respond to oxidative stress were found (97). The age-related decrease in the ability to mount a ubiquitin-dependent response upon oxidation may contribute to the accumulation of damaged proteins in the old lenses.

Other proteolytic systems also decline with age in lens. Studies in cultured epithelial lens cells revealed that, after successive passages, there was a decrease in the proteolytic response to serum removal. This degradative defect mainly affects proteolysis of long-lived proteins suggesting that the degradative defect may be related with the hsc73-mediated lysosomal pathway of protein degradation (208).

6. CONCLUSIONS AND PERSPECTIVES

Many of the changes that happen in senescent cells can be explained based on reduced rates of intracellular protein degradation. The decrease in total protein breakdown with age has been extensively reported, and the specific intracellular proteolytic systems responsible for this decrease are now becoming clear. Dietary restriction extends the lifespan of rats, and this treatment also delays the age-related decline in liver protein degradation (195), consistent with reduced protein degradation being an important parameter in aging.

We have presented a general review of age-related changes in different proteolytic systems. However, possible modifications in the proteolytic susceptibility of substrate proteins with age should also be considered, because both a decrease in the activity of proteolytic pathways and also a decrease in the proteolytic susceptibility of substrate proteins could result in accumulation of proteins in senescent cells. For example, certain oxidized proteins and cross-linked proteins are poor substrates for proteases and are slowly degraded by cells (188). This stabilization may contribute to the observed accumulation and damaging actions of oxidized proteins during aging (188). However, these reductions in proteolytic susceptibility with age do not seem to apply to the majority of protein modifications (for review see ref. 90). In fact, there is an increase in susceptibility to degradation by exogenous proteases of cytosolic proteins from liver of old rats (186), and from senescent fibroblasts (187), and it has been proposed that different age-specific

protein modifications must contribute to the increased susceptibility to proteolytic attack (186).

There are still many aspects of intracellular protein degradation that need to be addressed in order to identify the primary defects with age. The discovery of proteolytic activities in some cellular compartments until recently unknown (i.e. endosomes and mitochondria) has already expanded the roles of the protein breakdown inside cells. Identification of other new proteases might also explain the decrease in non-acidic protease activity in some models of senescence.

As described above, one of the principal pathways contributing to the age-related degradation failure is the hsc73-mediated lysosomal pathway. Several mechanistic aspects of this specific lysosomal pathway are unclear; the exact mechanism underlying cytosolic hsc73 stimulation of substrate binding to the lysosomal membrane receptor; the role of other cytosolic regulators of the hsc73 function; the existence of other lysosomal membrane and matrix protein(s) that participate in the transit of substrates into the lysosomal matrix. New substrates for this pathway need to be identified, and the contribution of this proteolytic system to the overall intracellular degradation in different organs and under different conditions must be further established.

The identification of proteolytic defects with age will also allow the development of different corrective methods to eliminate the cytosolic accumulation of abnormal proteins. One approach is to reduce protein damage (209). However, the use of protective enzymes is limited because cells are exposed to a large number of potential injurious agents. A more general solution for this problem will be to facilitate the elimination of the accumulated proteins.

We have previously demonstrated that the overexpression of the receptor protein for the hsc73-mediated lysosomal pathway in normal cultured cells results in an increase in the activity of this pathway (146). Regulated gene expression systems, such as the Tet-Off and Tet-On system regulated by tetracycline, are now available. Those systems allow a high-level expression of cloned genes in response to varying concentrations of tetracycline (210). The regulated expression of lgp96 in fibroblasts may allow us to increase the activity of the hsc73-mediated lysosomal pathway at different times of senescence. Thus, we could determined the reversibility of the age-related decrease in the activity of the selective lysosomal pathway, along with the effect of its activation in the elimination of the protein deposits in the cell. That procedure may result in a recovery of other normal cellular functions.

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