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Age-related disruption of autophagy in dermal fibroblasts modulates extracellular matrix components

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ABSTRACT

Autophagy is an intracellular degradative system that is believed to be involved in the aging process. The contribution of autophagy to age-related changes in the human skin is unclear. In this study, we examined the relationship between autophagy and skin aging. Transmission electron microscopy and immunofluorescence microscopy analyses of skin tissue and cultured dermal fibroblasts derived from women of different ages revealed an increase in the number of nascent double-membrane autophagosomes with age. Western blot analysis showed that the amount of LC3-II, a form associated with autophagic vacuolar membranes, was significantly increased in aged dermal fibroblasts compared with that in young dermal fibroblasts. Aged dermal fibroblasts were minimally affected by inhibition of autophagic activity. Although lipofuscin autofluorescence was elevated in aged dermal fibroblasts, the expression of Beclin-1 and Atg5—genes essential for autophagosome formation—was similar between young and aged dermal fibroblasts, suggesting that the increase of autophagosomes in aged dermal fibroblasts was due to impaired autophagic flux rather than an increase in autophagosome formation. Treatment of young dermal fibroblasts with lysosomal protease inhibitors, which mimic the condition of aged dermal fibroblasts with reduced autophagic activity, altered the fibroblast content of type I procollagen, hyaluronan and elastin, and caused a breakdown of collagen fibrils. Collectively, these findings suggest that the autophagy pathway is impaired in aged dermal fibroblasts, which leads to deterioration of dermal integrity and skin fragility.

1. Introduction

Autophagy is an evolutionarily conserved, intracellular degradation pathway that mediates the clearance of proteins, lipids, sugars and nucleic acids, as well as larger cellular components such as organelles. Three types of autophagy have been identified: microautophagy, chaperone-mediated autophagy (CMA), and macroautophagy [1]. Microautophagy is the least studied and occurs when cytoplasmic components are degraded following direct invagination, protrusion and/or septation of the lysosomal or endosomal membrane [2,3]. In CMA, a type of autophagy only present in higher organisms, the chaperone protein Hsc70 recognizes a specific pentapeptide motif (KFERQ) on unfolded proteins in the cytosol and escorts them directly to the lysosome-associated membrane protein type 2A (LAMP-2A). The cargo protein is then unfolded at the membrane and translocated into the lysosome for degradation [4]. Since current understanding of the regulation of microautophagy and CMA in relation to aging is limited, we primarily focused on macroautophagy in the present study.

Macroautophagy (hereafter referred to as autophagy) is a multistep process by which large macromolecules and organelles are engulfed in autophagosomes and degraded after fusion with lysosomes. Autophagy protects cells both constitutively and during cellular stress or starvation [5]. Thus, constitutive autophagy is important in the clearance of damaged/degenerated proteins or senescent organelles, and adaptive autophagy is crucial in the maintenance of cellular homeostasis during energy starvation or environmental stress. Besides its critical role in physiological conditions, dysregulated autophagy is implicated in diverse pathological processes such as neurodegenerative diseases, cancer, aging

Abbreviations: CMA, chaperone-mediated autophagy; LAMP-2A, lysosome-associated receptor protein type 2A; LC3, microtubule-associated protein 1 light chain 3; leu, leupeptin; MMP-1, matrix metalloproteinase-1; pep, pepstatin A.

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and infection [6]. However, the contribution of autophagy to age-dependent changes in human skin is unclear.

Skin aging is a continuous process that affects skin function and appearance. A reduction in the amount and organization of dermal components is believed to cause the main signs of skin aging, such as loss of elasticity, wrinkle formation and sagging. Damage to the skin through environmental exposure induces aging. The mechanism of skin aging has been well studied with a particular focus on the influence of photodamage (photoaging). Similar to other organs, the skin also undergoes chronological aging, which occurs in both sun-exposed and sun-protected areas [7]. Chronologically aged skin is characterized by a lower production of hyaluronan [8], elastin [9] and type I procollagen [10–12], and higher expression of matrix metalloproteinase-1 (MMP-1) [13]. Despite the documented age-dependent skin changes, the role of autophagy in chronological skin aging has not been examined. In this study, we examined the age-related changes of autophagy in dermal fibroblasts derived from elderly women and the effect of these changes on dermal components.

2. Materials and methods

2.1. Reagents and antibodies

Chloroquine was purchased from Nacalai Tesque (Kyoto, Japan). Leupeptin and pepstatin A were purchased from Sigma–Aldrich (St. Louis, MO). The hyaluronan assay kit was obtained from Seikagaku Biobusiness Corporation (Tokyo, Japan), the procollagen ELISA kit was obtained from Takara Bio (Shiga, Japan) and the MMP-1 assay kit was obtained from AnaSpec (Fremont, CA). Anti-LC3 antibody used for immunofluorescence was generated as described previously [32]. Anti-LC3 antibody used for Western blotting was purchased from Medical & Biological Laboratories (Nagoya, Japan). Anti-LAMP2 antibody was obtained from Abcam (Cambridge, UK). Goat anti-rabbit Alexa Fluor 488 was obtained from Molecular Probes (Carlsbad, CA).

2.2. Skin samples

Abdominal skin tissue from eight healthy Caucasian females (aged 25, 29, 35, 39, 45, 50, 55 and 60 years old) were purchased from Biopredic International (Rennes, France). The use of human skin samples was approved by the Institutional Ethical Committee of POLA Chemical Industries, INC. Sun-protected abdominal skin tissue was used to avoid the influence of photoaging as much as possible.

2.3. Cell culture

Normal cultured human dermal fibroblasts isolated from the abdomen of healthy Caucasian females were purchased from Biopredic International. Fibroblasts were cultured in Dulbecco's modified Eagle's medium (Sigma–Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum and antibiotics. The number of population doublings was calculated using the equation: population doublings = log 2 F/I, where "F" is the number of cells at the end of the passage and "I" is the number of cells seeded at the beginning of the passage. Subconfluent cultures that had undergone 20–30 population doublings were used for experiments.

2.4. Preparation of fibroblast-populated collagen lattice

Collagen matrix solution was prepared on ice by mixing 7 ml of 5 mg/ml bovine dermis-derived acid-solubilized type I collagen (Koken, Tokyo, Japan), 3.5 ml of fetal bovine serum, 7 ml of 5× Dulbecco's modified Eagle's medium (4500 mg/ml glucose without HEPES or $NaHCO_3$, Invitrogen), 3.5 ml of 200 mM HEPES, 3.5 ml of 2.2% $NaHCO_3$, 1.75 ml of 0.1 N NaOH and 5.25 ml of H_2O. The final concentration of collagen and FBS in the mixture was 1 mg/ml and 10% (v/v), respectively. A basal layer of collagen was made by adding 250 μl of the solution into individual wells of a 24-well plate followed by a 15-min incubation at 37 °C. A fibroblast-populated collagen layer was made by adding 1 ml of a mixture of 22.5 ml collagen matrix solution with 2.5 ml suspension of fibroblasts (1×10^5 cells/ml) onto the basal layer. The number of fibroblasts in each well was 1×10^5 cells. The collagen lattices were released from the wells with a needle 2 h after incubation and 0.75 ml of medium was subsequently added to the lattices (day 0). On days 1 and 3, medium was renewed using leupeptin/pepstatin-containing media. The lattices were then fixed in glutaraldehyde on day 4 and processed for SEM as described below.

2.5. Electron microscopy

For transmission electron microscopy (TEM), glutaraldehyde-fixed skin tissues were treated with 1% osmium tetroxide. Samples were dehydrated with graded ethanol and propylene oxide, and embedded in pure epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate, and observed in a Hitachi H-7100 Transmission Electron Microscope (Hitachi, Tokyo, Japan). For scanning electron microscopy (SEM), glutaraldehyde-fixed collagen lattices were dehydrated with graded ethanol and amyl acetate. Critical point drying was performed using liquid carbon dioxide. Specimens were then mounted on stubs using double-sided tape and coated with gold in a sputter coater. The specimens were examined using a JSM-6380LA microscope (JEOL, Tokyo, Japan).

2.6. Western blotting

Proteins in cell lysates were separated by electrophoresis in a 12.5% polyacrylamide gel (ATTO, Tokyo, Japan) and transferred to a PVDF membrane (Immobilon-P, Millipore, Bedford, MA). The membrane was incubated with appropriate primary and secondary antibodies following standard protocols. The immunoreactive bands were detected using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Yokohama, Japan) and visualized by densitometry (ATTO Densitograph version 3, ATTO, Tokyo, Japan).

2.7. Immunofluorescence microscopy

Cells cultured on coverslips were fixed with 4% paraformaldehyde in PBS, permeabilized with 0.005% digitonin in PBS, quenched with 50 mM NH_4Cl in PBS, and blocked with 1% bovine serum albumin in PBS. Cells were then incubated with the anti-LC3 antibody (1:400) followed by incubation with a secondary antibody, and PBS washes were performed after antibody incubations. Coverslips were mounted in Dapi-Fluoromount-G (Southern Biotech, Birmingham, AL) and observed using a confocal microscope (LSM 510 META, Carl Zeiss, Oberkochen, Germany).

2.8. Assay of proteasome activity

The assay was performed as previously described [32]. Briefly, harvested cells were lysed with a proteasome buffer (containing 10 mM Tris–HCl pH 7.5, 1 mM EDTA, 2 mM ATP, 20% glycerol, 4 mM DTT and 0.5% Triton X-100), sonicated, and then centrifuged at 13,000g at 4 °C for 10 min. The resulting supernatants (20 μg of protein) were incubated with proteasome activity assay buffer (containing 100 mM Tris–HCl pH 7.5, 1 mM ATP, 10 mM $MgCl_2$, 1 mM DTT and 100 μM Suc-Leu-Leu-Val-Tyl-4-methylcoumaryl-

7-amide [Suc-LLVY-MCA, Peptide Institute, Osaka, Japan]) in a total volume of 100 μl for 1 h at 37 °C. The reaction was stopped by adding 1 ml of 5% SDS. Proteasome activity was determined by measuring the fluorescence intensity (excitation at 380 nm and emission at 460 nm) of 7-amino-4-methylcoumarin, one of the reaction products, using fluorescence spectrophotometry (ARVO-SX, Perkin Elmer, Waltham, MA).

2.9. Quantitative RT-PCR

Quantitative RT-PCR was carried out using StepOnePlus Real-Time PCR System (Applied Biosystems) with QuantiFast SYBR Green PCR kit (Qiagen, Venlo, Netherlands) according to the manufacturer's instructions. All primers were obtained from Qiagen. Expression level of target genes was normalized to that of endogenous β-actin in each sample.

2.10. Detection of lipofuscin-like autofluorescence

Cells cultured on coverslips were fixed with 1% paraformaldehyde in PBS for 5 min and were mounted in Fluoromount-G (Southern Biotech). Autofluorescence of lipofuscin granules was then observed by excitation at 488 nm using a 590 nm barrier filter.

2.11. Statistical analysis

Tukey's test was used to compare differences between young and aged cells. Other experiments were analyzed using the Student's *t*-test. *P*-value of less than 0.05 was considered statistically significant. The *P*-value presented is for a two-tailed test.

3. Results

3.1. Autophagosomes accumulate in elderly skin

Age-dependent differences in autophagy in skin tissue taken from women of different ages were examined by TEM. In the process of autophagy, cytoplasmic proteins and organelles are engulfed by autophagy-specific structures in the cytoplasm known as autophagosomes (Fig. 1A). TEM findings revealed that the number of autophagosomes in dermal fibroblasts increased with age (Fig. 1B). No significant difference in the size of autophagosomes was observed between different samples (data not shown).

Autophagosome formation involves the conversion of cytoplasmic microtubule-associated protein 1 light chain 3 (LC3, mammalian ATG8) from a cytosolic (LC3-I) to a membrane-bound form (LC3-II) by conjugating to phosphatidylethanolamine via its C-terminus [14,15]. Immunofluorescence microscopy showed that

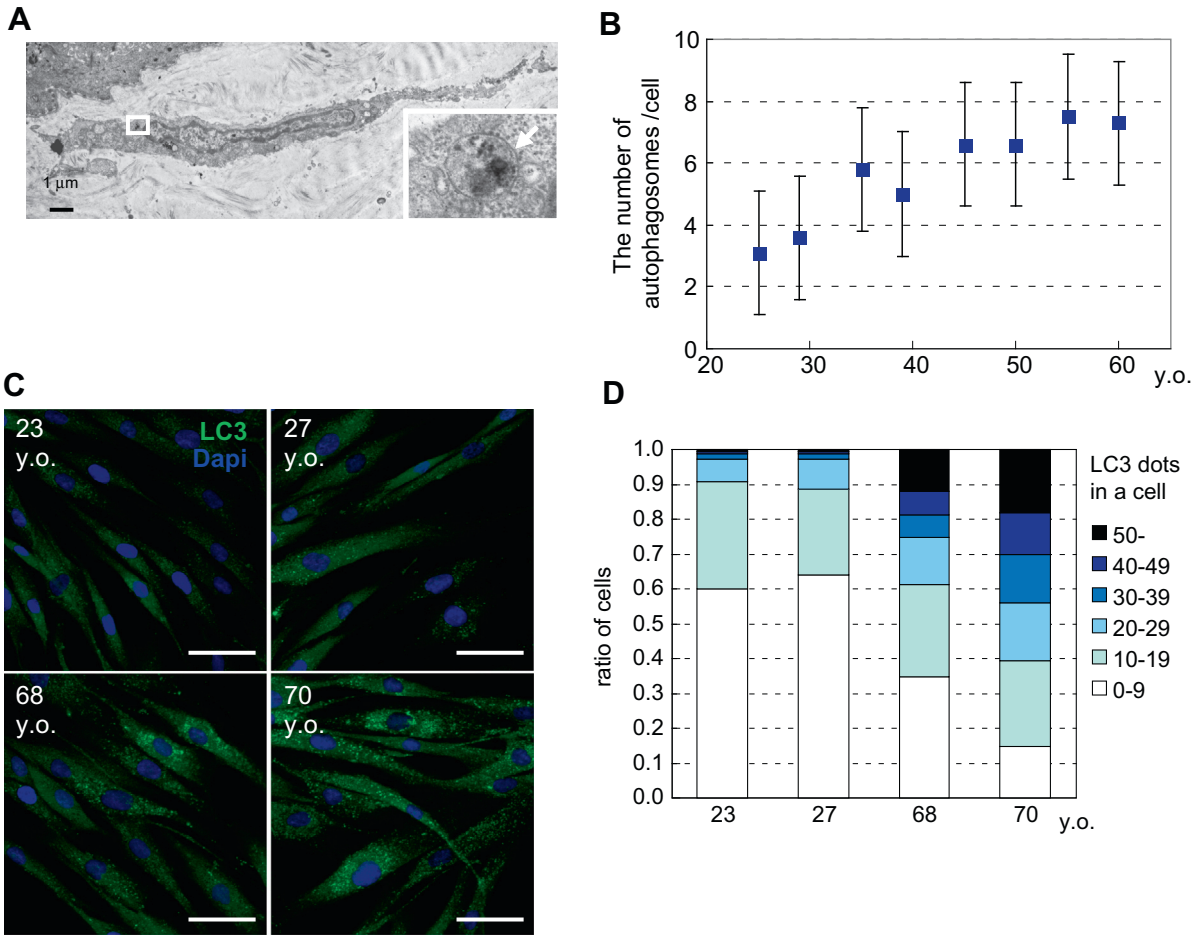

Fig. 1. Accumulation of autophagosomes in dermal fibroblasts derived from elderly skin. (A) A representative transmission electron microscope image of dermal fibroblasts in skin tissue taken from a 60-year-old woman. The inset is a magnified view of the boxed area, and the arrow points towards an autophagosome. (B) Ten cells in each skin tissue were examined and the number of autophagosomes per cell was calculated. Data represent the mean ± SD. Spearman's ρ is 0.9461. (C) Cultured dermal fibroblasts were fixed and stained with anti-LC3 antibody. Scale bar, 50 μm. (D) LC3 dots in each cell were counted. The proportion of cells with different numbers of LC3 dots is shown.

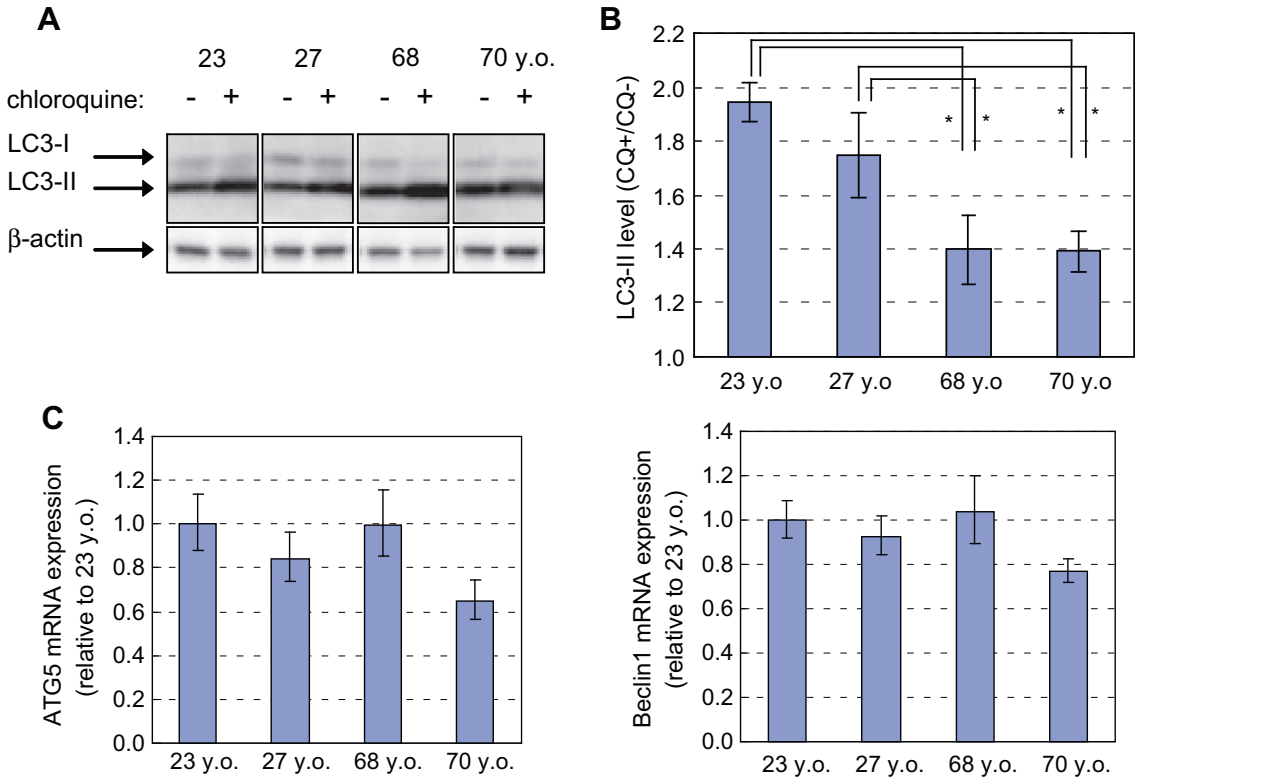

Fig. 2. Autophagic degradation is impaired in dermal fibroblasts derived from elderly women. (A) Cell lysates were subjected to SDS–PAGE and Western blotting. (B) Densitometry analysis was performed using Image J software (NIH) and data were normalized using β-actin. Data are expressed as mean ± SD of three separate experiments. (C) Expression of ATG5 and Beclin-1 mRNA was measured by quantitative RT-PCR. Data are expressed as mean ± SD of three separate experiments. The difference between all pairs was not significant (Tukey's test).

punctate LC3 staining was significantly greater in cultured dermal fibroblasts derived from the skin of elderly women (68 and 70 years old) (hereafter referred to as aged cells) compared with those derived from the skin of young women (23 and 27 years old) (hereafter referred to as young cells) (Fig. 1C and D). Similar results were seen in Western blot analysis of LC3-II levels in chloroquine-negative samples (Fig. 2A). These results indicated that age-dependent changes in autophagy occur in dermal fibroblasts.

3.2. Autophagy is disrupted at the degradation step in aged dermal fibroblasts

After complete formation of the autophagosome, it fuses with the lysosome that contains hydrolytic enzymes, resulting in the degradation of the sequestered material in the autophagosome. As the relative increase in the number of autophagosomes in aged cells may reflect either upregulated autophagosome formation or blockage of autophagic degradation [16], we first examined autophagic degradation by determining the levels of LC3-II in young and aged cells treated with chloroquine, an agent that impairs autophagic degradation by inhibiting lysosomal acidification and proteolysis. Western blot analysis revealed that aged cells had smaller increases in the level of LC3-II than young cells after chloroquine treatment (Fig. 2A and B), suggesting that the autophagic degradation is impaired in aged cells.

To test whether autophagosome formation is increased in aged cells, we examined the expression of Beclin-1 (mammalian ATG6) and ATG5 that are involved in autophagosome formation. Beclin-1 is required for the initiation of autophagosome formation while ATG5 forms a complex with ATG12 and ATG16L1 that is necessary for the autophagosomal elongation process. As shown in Fig. 2C, no significant differences in Beclin-1 and ATG5 expression were observed between young and aged cells, suggesting that the autophagosome accumulation observed in aged cells is unlikely to be associated with upregulation of autophagosome formation.

3.3. Lipofuscin-like autofluorescence accumulates in aged dermal fibroblasts

Impaired protein degradation causes a low turnover of proteins. Abnormal, damaged proteins may form aggregates because of aberrant intermolecular interactions, and form non-degradable lipofuscin after further oxidization and cross-linking [17]. Autofluorescence, which primarily originates from accumulated lipofuscin [18], accumulates in aged tissues such as the brain [19]. Replicative senescent fibroblasts also have elevated levels of autofluorescence [20]. In addition, it has been shown that knockdown of autophagy-related genes causes an increase of autofluorescence [21]. We examined whether autofluorescence is increased in dermal fibroblasts derived from elderly donors. As shown in Fig. 3A, a stronger autofluorescence was observed in aged cells compared with young cells. This suggests that the protein turnover in cultured dermal fibroblasts derived from elderly women was less than that in cultured dermal fibroblasts from young women, which led to lipofuscin accumulation.

3.4. Proteasome activity is not suppressed in aged dermal fibroblasts

The ubiquitin–proteasome and autophagy–lysosome pathways are the main routes of protein clearance in eukaryotic cells [22]. Previous reports indicated that the activity of the proteasome degradation pathway is suppressed in certain organs in elderly individuals [23]. Therefore, we checked whether proteasome activity

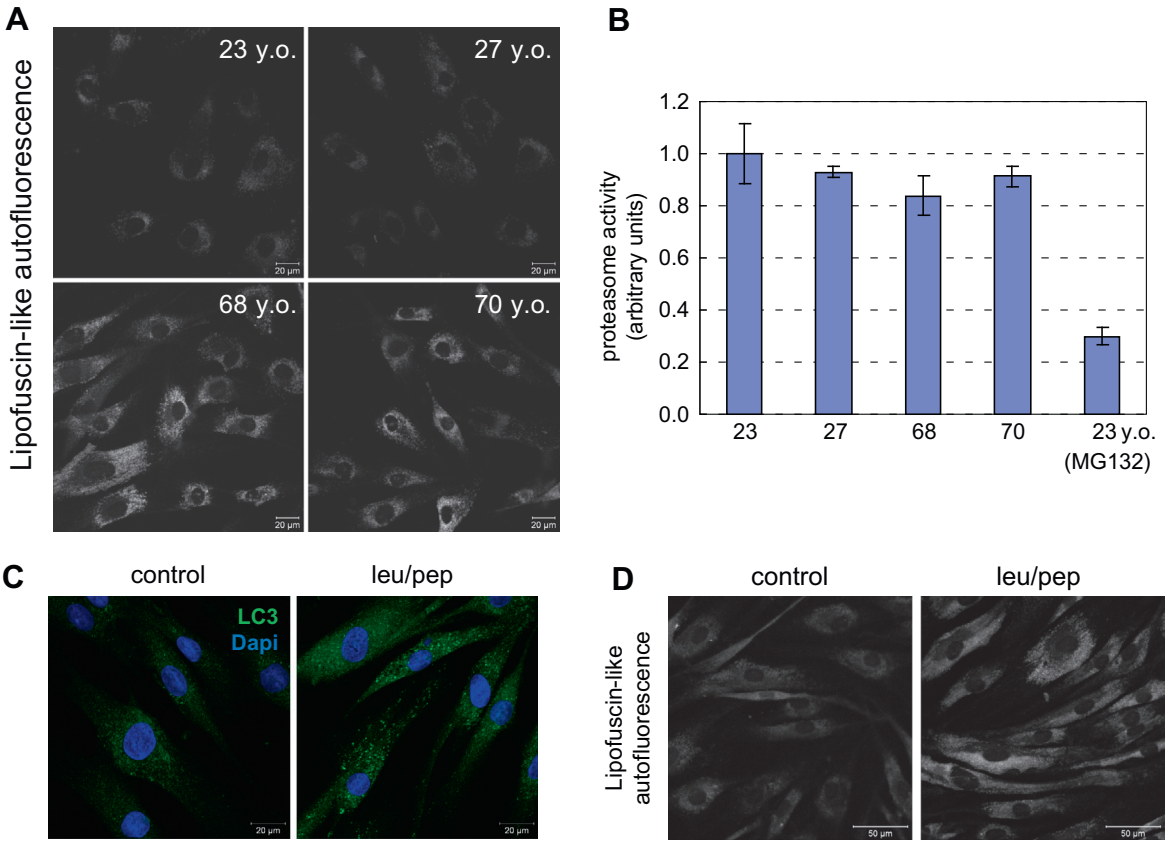

Fig. 3. Relative increase in lipofuscin-like autofluorescence in dermal fibroblasts derived from elderly women secondary to autophagy disruption. Leupeptin and pepstatin A (leu/pep)-treated young dermal fibroblasts display features of elderly dermal fibroblasts characterized by autophagosome and lipofuscin accumulation. (A) Lipofuscin-like autofluorescence in dermal fibroblasts derived from women of different ages was examined by confocal microscopy. (B) The rate of Suc-LLVY-AMC degradation was measured in lysates of dermal fibroblasts derived from women of different ages. Relative proteasome activity was compared with that of cells from a 23-year-old woman. MG132, an inhibitor of proteasome activity, was used to ensure the specificity of the assays. Data are expressed as mean ± SD of three independent experiments. (C) Non-treated and leu/pep-treated cells were stained with anti-LC3 antibody. (D) Lipofuscin-like autofluorescence in dermal fibroblasts derived from a 23-year-old woman was examined by confocal microscopy.

Table 1
Changes in dermal components after leu/pep treatment.

Dermal components		Amount in leu/pep-treated cells (relative to control)
Type I procollagen	mRNA	0.799 ($p = 0.099$)
	Protein	0.661 ($p = 0.023$)
Hyaluronan	–	0.373 ($p < 0.001$)
Elastin	mRNA	0.553 ($p = 0.005$)
MMP-1	mRNA	2.025 ($p = 0.001$)
	Activity	1.328 ($p = 0.003$)

is different among dermal fibroblasts from women of different ages. As shown in Fig. 3B, no marked difference in proteasome activity between young and aged cells was observed, indicating that the accumulation of lipofuscin in aged cells was not due to impaired proteasome activity. These data suggest that impairment of the autophagic pathway rather than the proteasome pathway caused age-related lipofuscin accumulation in dermal fibroblasts.

3.5. Influence of autophagy disruption on the dermis

To investigate whether impairment of the autophagic pathway leads to age-related changes in the dermis, young cells were trea-

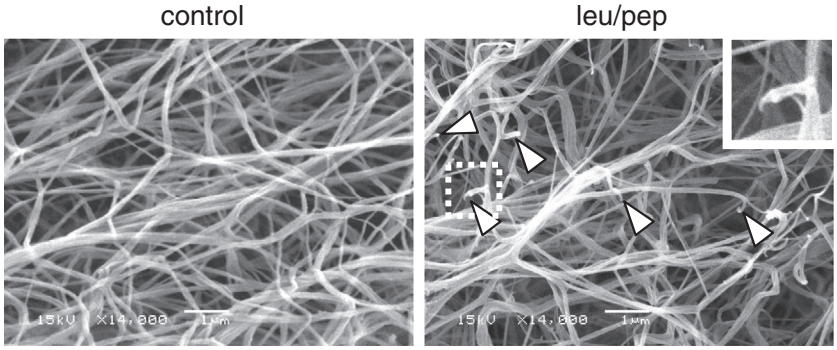

Fig. 4. Broken collagen fibrils were observed in the collagen lattice populated with leu/pep-treated cells. Collagen lattices were examined by scanning electron microscopy. Arrowheads point to broken fibrils. The inset in the upper right-hand corner is a magnified view of the boxed area.

ted with leupeptin and pepstatin A (leu/pep)—major lysosomal protease inhibitors—to mimic autophagy-suppressed aged cells. Both the number of LC3-positive vesicles (Fig. 3C) and the intensity of autofluorescence (Fig. 3D) were significantly increased in young cells treated with leu/pep, which resembled the condition seen in aged cells. Moreover, leu/pep-treated cells displayed diminished levels of type I procollagen, hyaluronan and elastin, and elevated levels of MMP-1 (Table 1). In particular, MMP-1, which induces collagen fragmentation [11], showed a significant increase in both expression and activity after leu/pep treatment. SEM revealed broken collagen fibrils in the collagen lattice populated with leu/pep-treated cells (Fig. 4). These results suggest that impaired autophagic flux caused a decrease in dermal components and collapse of dermal structure, which leads to skin fragility.

4. Discussion

The present study focused on age-related changes in the autophagic process in the dermis. We found that the amount of autophagosomes in dermal fibroblasts from the skin of elderly Caucasian females was comparatively elevated. In vitro experiments showed that the degradation step of autophagy was suppressed in dermal fibroblasts derived from elderly donors, which led to autophagosome accumulation.

It has been reported that the autophagic process in the specific tissues in mammals is affected by age. For example, autophagy function and activity decrease with age in the rodent liver [24] and in hypothalamic neurons in mice [25]. To the best of our knowledge, the present study is the first to demonstrate age-related changes in autophagy in human dermal fibroblasts by showing autophagosome accumulation in cultured dermal fibroblasts and in dermal fibroblasts in situ in skin samples from elderly women. Although a previous report has described the increase in lipofuscin, the so-called "aging pigment", in replicative senescent cells, our study is the first to demonstrate the lipofuscin increase in dermal fibroblasts derived from elderly donors.

Autophagosome accumulation has been detected in neurons of neurodegenerative diseases including Alzheimer's disease, transmissible spongiform encephalopathies, Parkinson's disease and Huntington's disease (reviewed in [26,27]). The abnormal accumulation of autophagosomes is thought to contribute to impaired cellular/tissue function. It is possible that the autophagosome accumulation in aged dermal fibroblasts contributes to skin aging.

The role of autophagy in the skin is poorly understood, especially in the dermis. In epidermal keratinocytes in vitro, autophagy is upregulated by stimuli and processes such as terminal differentiation [28], metabolic stress [29], calcipotriol, a Vitamin D analog [30], and activation of Toll-like receptors [31]. These findings led to the hypothesis that autophagy is involved in cornification [28,29]. In the present study, we investigated the influence of age-related changes in autophagy on extracellular matrix components that regulate the structure of the dermis. Results showed that suppression of autophagic degradation using lysosomal enzyme inhibitors decreased the levels of collagen, elastin and hyaluronan, and increased expression/activity of the collagen-degrading enzyme MMP-1. Changes in collagen, elastin and hyaluronan may reduce dermal stiffness and lead to the development of wrinkles and sagging in aged skin. In addition, the changes caused by suppression of autophagic degradation coincide with those reported in chronologically aged skin. Taken together, our study findings suggest that age-related impairment of autophagy is involved in the onset and progression of aging in the dermis.

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