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Accelerated tubular cell senescence in SMP30 knockout mice

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Summary. An experimental model with accelerated but not drastic renal senescence seemed useful to recognize the mechanisms of how kidney function deteriorates with age. Senescence marker protein-30 (SMP30), whose expression decreased with age and was sex-independent, is mainly expressed in hepatocytes and proximal tubular cells. Therefore, we established a SMP30 deficient strain of mice with a C57BL/6 background by gene targeting to investigate whether this molecule is involved in renal tubular cell senescence. Male SMP30 knockout (SMP30Y/-) mice and male wild-type (SMPY/+) mice (n=5) aged 12 months were examined histologically. Their tubular epithelia showed the deposition of lipofuscin and the presence of senescence-associated ß-galactosidase (SA-ß-GAL). However, no tubular cells were atrophic. In electron microscopy, SMP30-KO mice showed markedly enlarged lysosomes containing an electron dense substance. These are convincing hallmarks of senescence. We recognized the early manifestation of senescence hallmarks in SMP30-KO mice at 12 months old. Thus, this model represents the first report of a mouse strain that manifests accelerated ordinal senescence in a kidney after gene manipulation.

Key words: Renal senescence, Tubular cells, SMP30, Knockout mouse, Lipofuscin, Senescence-associated, ß-galactoside

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Introduction

Aging results in profound anatomic and functional deterioration in renal systems both in humans (Davies and Shock, 1950; Hoang et al., 2003; Melk et al., 2004) and in animals (Yumura et al., 1989; Melk et al., 2003). These changes increase the risks for acute renal failure or chronic renal failure. In addition, kidney transplantation from the elderly performed poorly (Moreso et al., 1999; Kasiske and Snyder, 2002). Therefore, much attention has been devoted to studies of aging kidney. Renal senescence is a pleiotropic phenomenon induced by both intrinsic and extrinsic factors. This phenomenon appears gradually but inevitably as every individual ages. Seeking influential factors that induce senescence is a compelling subject.

In contrast, considerable evidence has accumulated of the molecular contribution to senescence in mitotic cells. Cultured mammalian somatic cells, such as fibroblasts, after a finite number of population doublings, eventually reach a state in which they irreversibly cease replication and manifest abnormalities (Hayflick and Moorhead, 1961; Wright and Shay, 2002). This state has been called replicative or cellular senescence. Senescent cells are identified in culture by their failure to synthesize with passage. In vitro, however, cell growth is not easily manipulated or monitored, and measurements of DNA synthesis do not distinguish senescent cells from quiescent or terminally differentiated cells. Dimri et al. reported that senescence-associated b-galactosidase (SA-ß-GAL) could be a good marker of replicative senescence. An age-dependent manifestation of SA-ß-GAL in human skin is the accumulation of senescent fibroblasts and keratinocytes *in vivo* (Dimri et al., 1995). In addition, lipofuscin is, apparently, a universal feature of aging (Harman, 1989).

Numerous molecules are associated with senescence. During a survey of such molecules by proteomic

analysis, we discovered a novel molecule in the rat liver (Fujita et al., 1992). Its expression decreased with age and was sex-independent. We designated this molecule as senescence marker protein-30 (SMP30). Phylogenically, the amino acid sequence of this molecule was highly conserved among all animals examined (not published). However, the function of this molecule is not entirely clear. Subsequently, we established a SMP30 deficient strain of mice with a C57BL/6 background by gene targeting (Ishigami et al., 2002). This strain is very sensitive to apoptosis induced by anti-Fas antibody or TNF-α and the lack or decrease of SMP30 seemed to cause organ frailty with aging. Recently we observed that the life span of SMP30-knockout (KO) mice was shorter than that of the wild type strain (Ishigami et al., 2004). Although SMP30 is expressed in almost all organs, the prominent sites expressing this molecule are the liver and kidney (Fujita et al., 1992, 1999). The proximal region of tubular epithelial cells expresses SMP30 abundantly. Since the deficiency of SMP30 in these KO mice can be regarded as the ultimate decrease, one can expect that they will undergo substantial organ deterioration with aging.

An experimental model with accelerated but not drastic senescence seemed useful not only to recognize the mechanisms of how kidney function deteriorates with age but also to use studies for disease susceptibility of aging kidneys. The desirable animal model in which to study kidney aging is one that manifests hallmarks of senescence at an early stage.

Materials and methods

Animals

The SMP30 knockout mice with C57BL/6 background were generated by gene targeting (Ishigami et al., 2002). In the present study, we used male SMP30 knockout (SMP30Y/-) mice (n=5) and male wild-type (SMP30Y/+) mice (n=5) aged 12 months. Mice were maintained at 12 hours day/dark cycles in a controlled environment and fed ad libitum. The Animal Care and Use Committee of Tokyo Metropolitan Institute of Gerontology approved the protocol of the animal experiment performed in the present study. To obtain renal tissues, mice were exsanguinated via abdominal aorta under anesthesia with intraperitoneal injection of pentobarbital (10 mg per 100 g body weight) and perfused via portal vein with phosphated-buffered saline (PBS).

Histological examination

Paraffin embedded specimens were cut at 2 μm and stained with periodic acid-Schiff for histopathological assessment. For electron microscopy, mouse kidneys were fixed with 2.5% glutaraldehyde in PBS. The specimens were post-fixed in 1% osmium tetroxide, dehydrated in a graded alcohol series, and embedded in epoxy resin. Semi-thin sections (1 μm) were stained with toluidine blue and examined under a light microscope. Then, ultrathin sections were prepared for double staining with urayl acetate and lead citrate; samples were then viewed under a Hitachi 100 electron microscope (Hitachi High-Technologies, Japan).

Senescence-associated (SA) ß-galatosidase (ß-GAL) staining

SA-ß-GAL staining was done by Senescence Detection Kit (BioVision Research Products, Mountain View, CA). The kidney sections embedded in OCT compound were fixed with Fixative solution for 10 minutes at room temperature, and then washed 3 times with PBS. The sections were incubated with Staining Solution Mix overnight at 37°C. Then, the sections were counterstained with hematoxylin and eosin. Positive reaction was detected as a blue color under light microscopy.

Results

Accumulation of lipofuscin in proximal tubular cells was accelerated in SMP30-KO mice

Light microscopically, glomeruli of SMP30-KO mice at 12 months old showed normal appearance comparable to wild type mice. Tubular atrophy, interstitial fibrosis, and atherosclerosis were not observed in all examined mice. The prominent morphological feature of kidneys from SMP30-KO mice was a massive accumulation of lipofuscin (age pigment) in proximal tubular epithelial cells (Fig. 1a,b), and these granules were predominantly observed in S2 or S3 segments. Lipofuscin accumulation in tubular cell was detected in all SMP30-KO mice examined at 12 moths old. However, kidneys from the wild type mice contained little lipofuscin (Fig. 1c,d).

Expression of senescence-associated ß-galactosidase in proximal tubular cells increased in SMP30-KO mice

Another hallmark of senescence is SA-ß-GAL (Morreau et al., 1989; Campisi, 1996). In renal tissues from SMP30-KO mice, SA-ß-GAL staining was observed only in proximal tubular cells, but not in glomeruli or vessels (Fig. 2a,b). However, in wild type no SA-ß-GAL was found (Fig. 2c,d). Although we have noted that lipofuscin is always deposited with SA-ß-GAL, some tubular epithelial cells without detectable lipofuscin deposition were positive for SA-ß-GAL in samples from the SMP30-KO mice.

Enlarged lysosomes were present in tubular cells of SMP30-KO mice

Ultrastructural study of kidneys from SMP30-KO mice showed markedly enlarged lysosomes containing

an electron dense substance in their tubular cells (Fig. 3). On the other hand, lysosomes in tubular cells of wild type appeared normal (not shown).

Discussion

In humans, various types of progeria caused by genetic disorder were reported. The extent of symptoms varies in each progeria. The phenotypes of such disorders usually differ from those of ordinal senescence. Consequently, human progeria has not inspired an appropriate experimental model available by gene manipulation. Some strains of mice having extended life spans or extreme acceleration of senescence have been reported (Kuro-o et al., 1997; Migliaccio et al., 1999), but the mechanisms elucidated by those systems are not always applicable to ordinal senescence. Furthermore, to establish an appropriate experimental model for aging research, some consensus on the criteria of accelerated aging is required. On the basis of previous reports by many investigators, the accelerated deposition of lipofuscin and SA-ß-GAL can

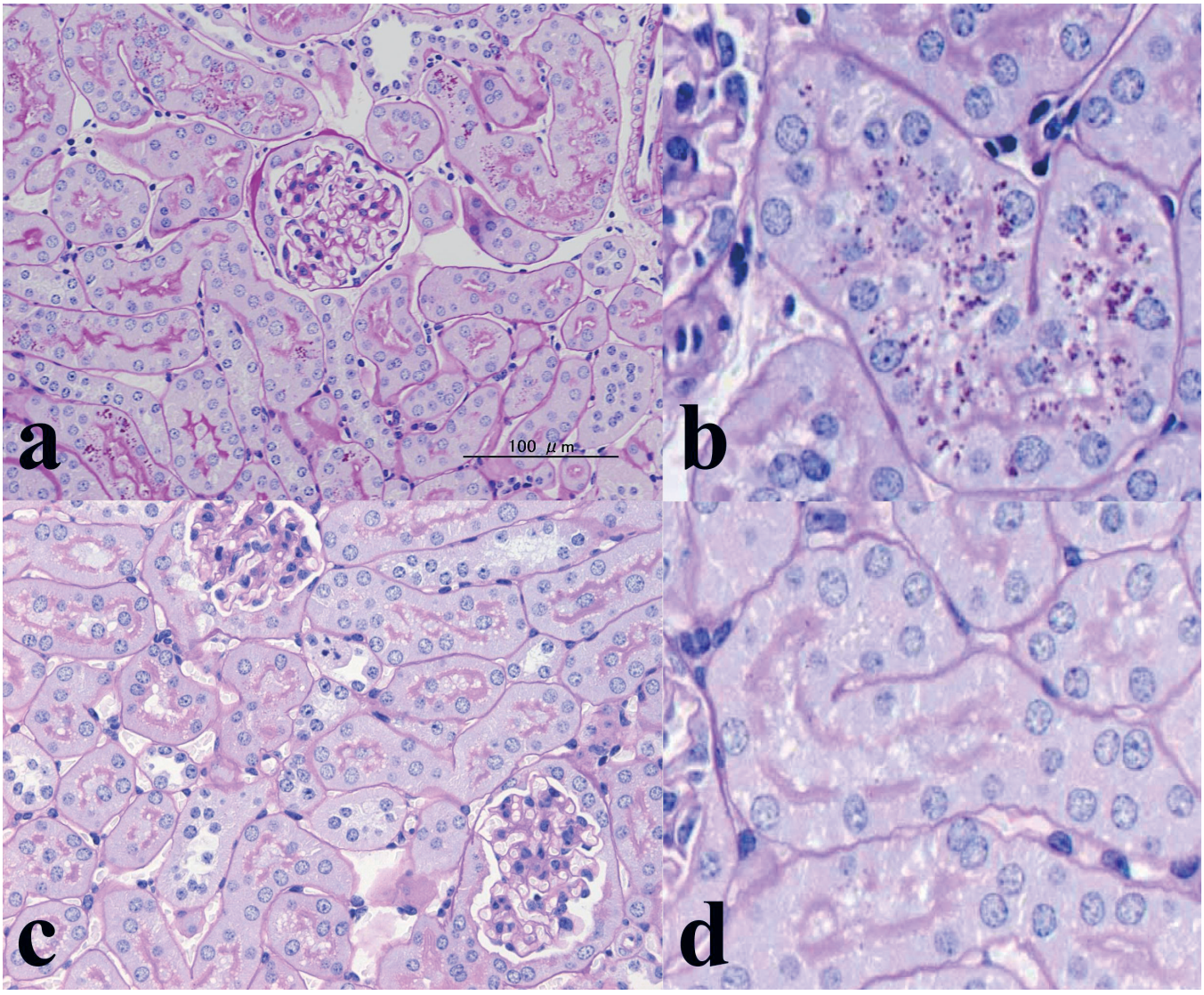

Fig. 1. Lipofuscin deposits accumulate abundantly in renal tubular epithelia of 12-month-old SMP30-KO mice. Paraffin-embedded tissue fixed in 10% formalin were cut at 2 μm and stained with periodic acid-Schiff for histopathological assessment. **a and b.** In the section from a 12-month-old SMP30-KO mouse, numerous brown granules were identified in proximal tubular cells as lipofuscin. **c and d.** In a wild type (SMP30-WT) mouse, kidney section contained very few lipofuscin granules. There were no tubular atrophy, interstitial injuries, and atherosclerosis in either group. Glomeruli also showed normal appearance. a,c x 200; b,d, x 400

serve as an adequate standard for measuring senescence.

In the present study we recognized the early manifestation of both senescence markers in the tubular epithelia of SMP30-KO mice. SMP30-KO mice showed marked deposition of lipofuscin (Fig. 1a,b). In contrast, lipofuscin deposits were barely detectable in comparable wild-type mice (Fig. 1c,d). Melk et al. reported that marked deposition of lipofuscin was present mainly in proximal tubules of aged rats and the largest amounts of lipofuscin lay in atrophic cells (Melk et al., 2003). Although SMP30-KO mice also had tubular cells with lipofuscin, there were no atrophic tubular cells in spite of the lipofuscin accumulation. This indicates that lipofuscin deposition precedes tubular atrophy. Apparently, then, SA-ß-GAL formation precedes lipofuscin deposition in SMP30-KO mice (Fig. 2). Thus the deposition of lipofuscin and SA-ß-GAL expression can be regarded as early parameters of organ senescence in our KO mice. Many reports described that proteinuria induce tubular injury (Chen et al., 1997). However, no albuminuria was detected in all SMP30-KO mice by single radial immunodifusion (data not shown). Therefore, these characteristics of tubular cell in SMP30-KO mice were not associated with proteinuria.

Brunk proposed the mitochondrial-lysosomal axis theory of aging (Brunk and Terman, 2002). According to

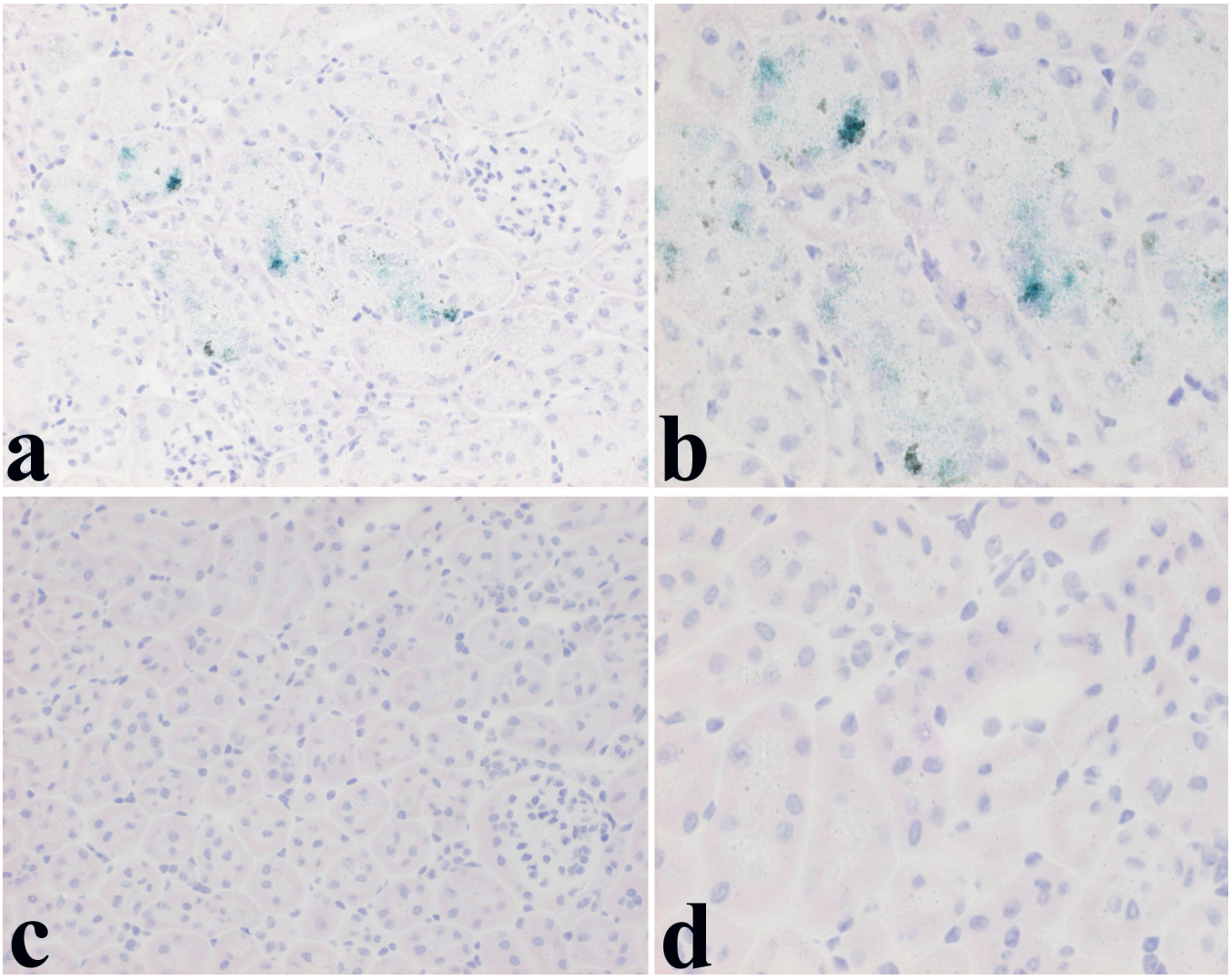

Fig. 2. a and b. SA-ß-GAL staining is positive in kidney tissue from 12-month-old SMP30-KO mice. SA-ß-GAL expression in the kidney section from a 12-month-old SMP30-KO mouse was detected only in proximal tubules, not glomeruli or vessels. **c and d.** Hematoxylin and eosin staining revealed lipofuscin deposition in SA-ß-GAL-positive tubular cells. Tubular cells of a 12-month-old wild type mouse barely expressed SA-ß-GAL. a, c, x 200; b, d x 400

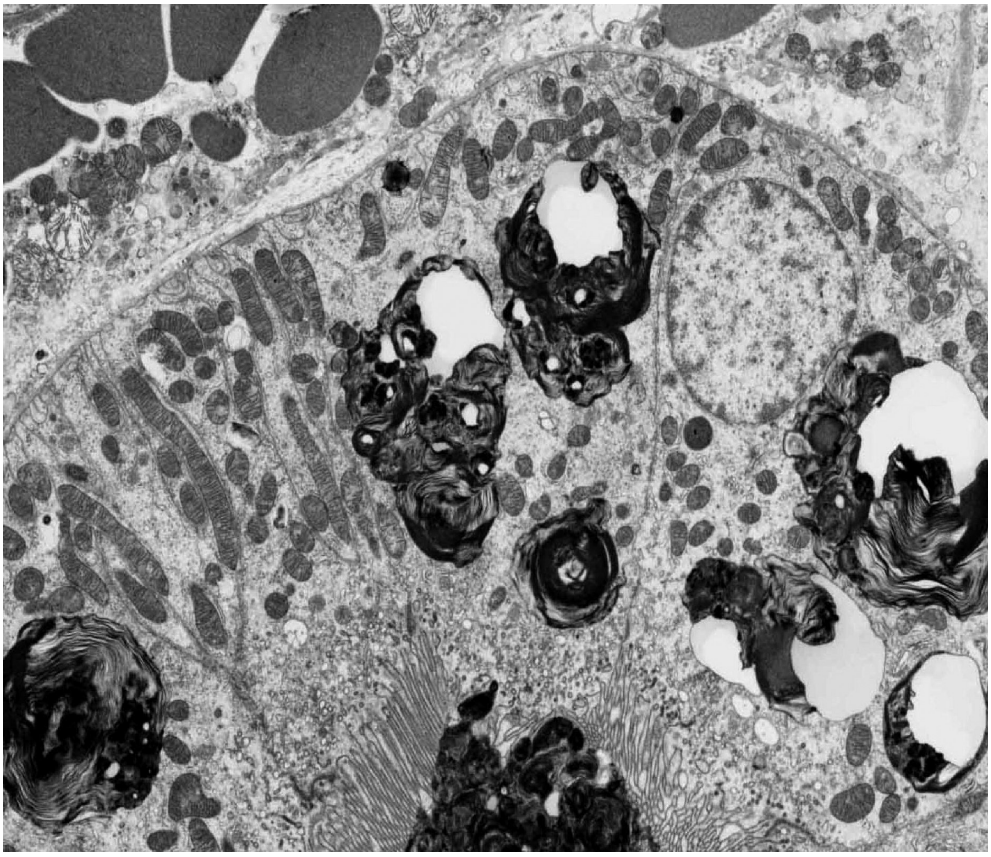

Fig. 3. Ultrastructural analysis revealed greatly enlarged lysosome containing an electron dense substance in a SMP30-KO mouse. x 800

that theory, age-associated accumulations of damaged mitochondria result from imperfect autophagocytosis. We did not observe marked mitochondrial decay in the present study. However, our previous examination of submandibular glands in 12-month-old SMP30-KO mice showed a high proportion of large mitochondria (Ishii et al., 2002). Ultrastructural changes of those membranes ranged from swelling and loss of cristae to complete deterioration and homogenization. Such morphological changes are associated with impaired fission. Additionally, abnormally enlarged mitochondrias are less likely to be autophagocytosed and recycled than those of normal size, leading to further mitochondrial damage. The mitochondrial decay observed in submandibular glands of SMP30-KO might be duplicated in the kidneys of much more aged individuals. We noted pronounced lysosomal enlargement along with extensive lipofuscin deposition in SMP30-KO mice (Fig. 3). Oxidative modification occurs primarily during autophagocytotic degradation inside lysosomes. Lipofuscin seems to undergo maturation reactions and form aggregates that finally may take over whole lysosomes. The process identified here corresponds with the mitochondrial-lysosomal axis theory of aging.

This is the first report of a mouse strain that manifests an acceleration of ordinal senescence after gene manipulation. This strain is expected to have many applications and holds particular promise for locating the missing-link between SMP30 deficiency and hallmarks of senescence.

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