male ICR mice. Also, cultured Ac2F liver cells were treated with *t*-BHP. Results in Figures 1C and 1D show that the decreased SMP30 expression correlated dose-dependently with *t*-BHP-induced oxidative stress.

Effect of age-related oxidative stress on transcriptional regulation of SMP30

Tests were performed to ascertain whether the downregulation of SMP30 may be due to reduced transcriptional regulation. Since the SMP30 promoter region contains many transcription binding sites, including Sp1, GATA factor, and newly discovered novel transcription binding sites, they were also examined.

Figure 2 shows the binding sites of transcription factors. To examine how aging and CR can affect DNA binding activities, EMSA was performed using rat nuclear protein. Sp1 and GATA factors were compared against the DNA binding activities at sites that are redox-sensitive transcription factors. Furthermore, as shown in Figure 2A, DNA binding activities at sites 3 and 5 decreased more significantly with age compared to sites 4 and 6. Figure 2B on the DNA binding activities of GATA and redox sensitive Sp1 shows a comparison with the effect of age and CR among sites 3, 4, 5, and 6. However, the DNA binding activities of GATA and Sp1 showed no significant age-related changes in relative to the DNA binding activities of sites 3 and 5, raising the possible implication of site 3 and 5. The sensitivity of SMP30 transcription to oxidative stress was detected by the LPS-induced treatment, which causes oxidative stress. We used EMSA to explore the exaggerated effect of LPS-induced oxidative stress on DNA binding activity during aging. As shown in Figure 2C, although the DNA binding activities of sites 3 and 5 decreased in the old group, the decrease in the LPS-challenged group was much more severe during aging. These results showed that transcriptional binding activities of SMP30 significantly decreased by an aggravated response from age-related and LPS-induced oxidative stress. However, as Figure 2C shows, changes in the DNA binding activities of sites 4 and 6 by the LPS-challenge showed no clear decreases. GATA was shown as comparing it to unidentified transcription factors. Therefore, results indicate that DNA binding activities of sites 3 and 5 are redox-sensitive.

Effects of oxidant t-BHP and antioxidant NAC on DNA binding activities of unidentified transcription factors in vitro

Binding activities at sites 3 and 5 are influenced by increased RS during aging (Figs. 2A and 2C). To verify more directly the effect of oxidative stress on sites 3 and 5, *t*-BHP treatment was used in nuclear protein to observe any changes in DNA binding activities of unidentified factors *in vitro*. As Figure 3A shows, DNA binding activities at sites 3 and 5 decreased in a dose-dependent manner with *t*-BHP treatment, but sites 4 and 6 and GATA changed little (Fig. 3B). Thus, results show that sites 3 and 5 seem sensitive to oxidative stress. To further explore the finding of the *t*-BHP effect, we used an inhibitor of oxidative stress, the potent antioxidant, NAC, to block the *t*-BHP-modulated binding activities of sites 3 and 5, confirming data in Figure 3C. Results in Figure 3C showed that antioxidant, NAC treatment dose-dependently ameliorates the decreased binding activities of sites 3 and 5.

Evidence for the effects of oxidant t-BHP *and ERK inhibitor in a cultured cell system*

To verify effects of oxidative stress and an antioxidant on SMP30 gene expression and the DNA binding activities of sites 3 and 5, we compared the actions of *t*-BHP and NAC on DNA binding activities in a cell nuclear extract. Cells were incubated with *t*-BHP and NAC during 1 h for separation of nuclear fraction and 5 h for separation of cytoplasmic fraction, respectively. Data in Figure 4A shows that decreased SMP30 expression by *t*-BHP was blunted by the addition of NAC. DNA binding activity of sites 3 and 5 showed a similar tendency in the expression of SMP30. In view of these results, age-related oxidative stress is responsible for the decline in SMP30 with age.

To determine the effects of oxidative stress and potentiality the phosphorylation of NF-κB and MAPK on SMP30 gene expression and DNA binding activities of sites 3 and 5, treatments with kinase inhibitors and *t*-BHP were tested. Cells were incubated with *t*-BHP and several kinase inhibitors, Bay 11–7085, PD098059, SB203580, and Wortmannin (specific inhibitors for NF-κB, ERK, MAPK, and phosphatidylinositol 3-kinase (PI_3K), respectively) for 1 h to separate the nuclear fraction or for 5 h to separate the cytoplasmic fraction. Figure 5A reveals that SMP30 expression decreased from the *t*-BHP treatment, whereas the cells treated with the ERK specific inhibitor, PD098059, recovered significantly from the decreased SMP30 brought about by *t*-BHP. As shown in Figure 5B, DNA binding activity at sites 3 and 5 decreased with *t*-BHP treatment, and was reversed with PD098059 treatment. These results revealed that the oxidative stress-induced decrease of DNA binding activity at sites 3 and 5 are likely regulated by the ERK pathway.

DISCUSSION

The current study was undertaken to elucidate the transcriptional regulation of SMP30 under increased oxidative stress during aging. Our data showed that the decline in the binding activity is due mainly to an inability of sites 3 and 5 to bind with nuclear transcriptional factors, which are influenced by redox status. Our findings also suggested that the declined transcriptional regulation of SMP30 is regulated by the ERK pathway. Based on these new revelations, we constructed a schematic model of SMP30 in Figure 5.

Supakar *et al.* (32) reported that an −800 bp upstream SMP30 promoter region and several novel DNA binding sites of transcription factors. To characterize the relation between senescent-related oxidative stress and SMP30 gene expression as a senescence marker protein, we studied the DNA binding activities of unknown transcription factors. The results from our current study affirmed the importance of the binding activities at sites 3 and 5 in relation to the transcriptional regulation of SMP30 as revealed by EMSA data. The results showing no correlation between GATA DNA binding

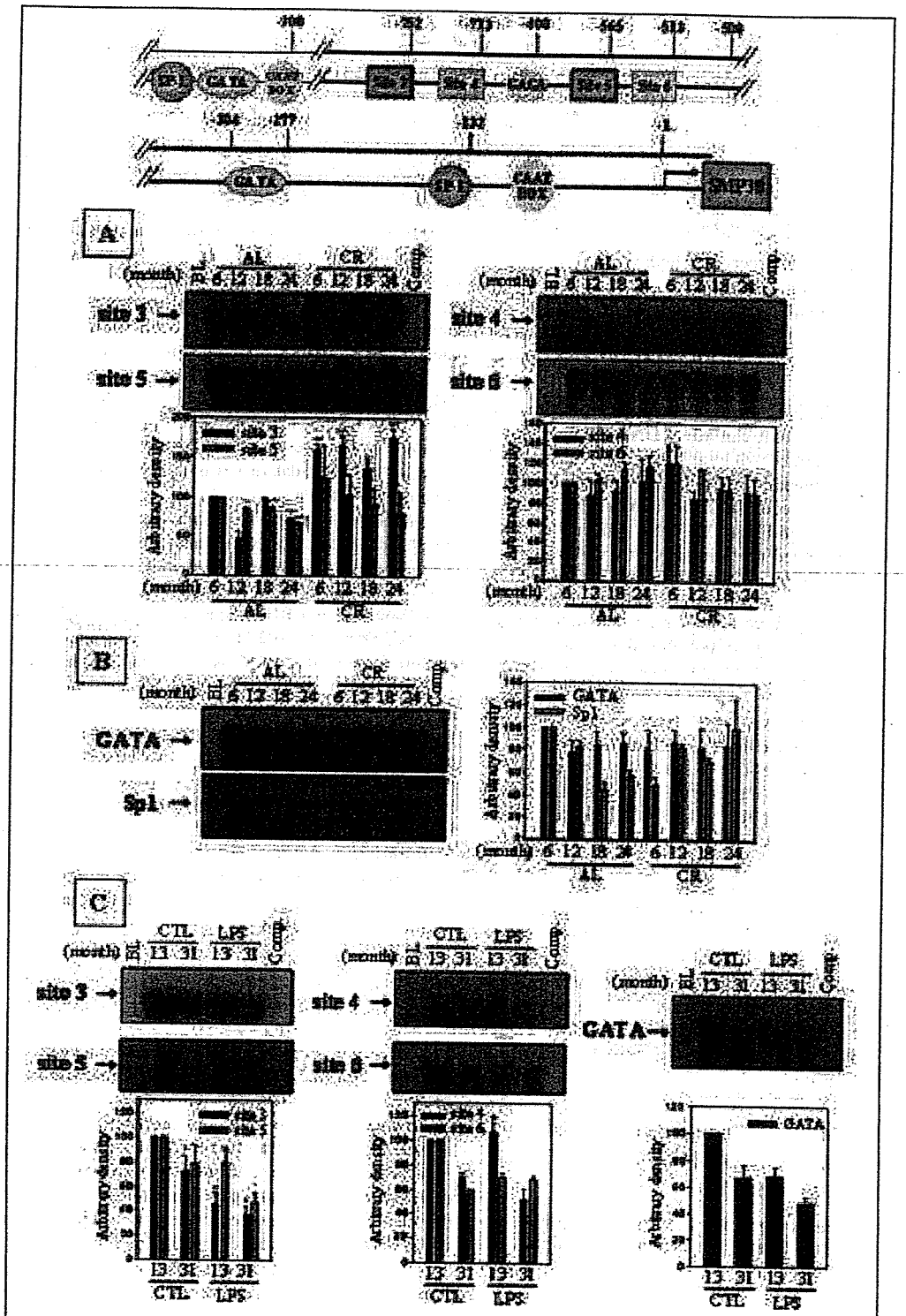

FIG. 2. Effects of age, CR, and LPS on DNA binding activities of unidentified transcription factors. Nuclear protein extracts (15 μg protein) of each group were incubated with ^{32}P-end-labeled probes containing a binding site (see Table 1). Binding activities of unidentified transcription factors are shown in (**A**) and announced transcription factor, GATA and Sp1 in (**B**) compared with unidentified transcription factors. (**C**) Nuclear extracts from rat liver administrated with LPS (5 mg/kg) at 13 and 31 months old were resolved by EMSA. In the present hypothetical study, the most obvious change was shown at sites 3 and 5. AL, *ad libitum* group; CR, calorie restricted group; CTL, vehicle-treated control group; LPS, LPS-injected group; BL, blank as no addition of nuclear fraction; comp., competitive confirmation with content of excess unlabeled oligonucleotides (200-fold competitor). As statistical significance, results of one factor ANOVA: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ *vs.* 6-month-old-rats of AL group or 13-month-old rats of CTL group; $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$ versus the same aged AL rats or CTL group, respectively.

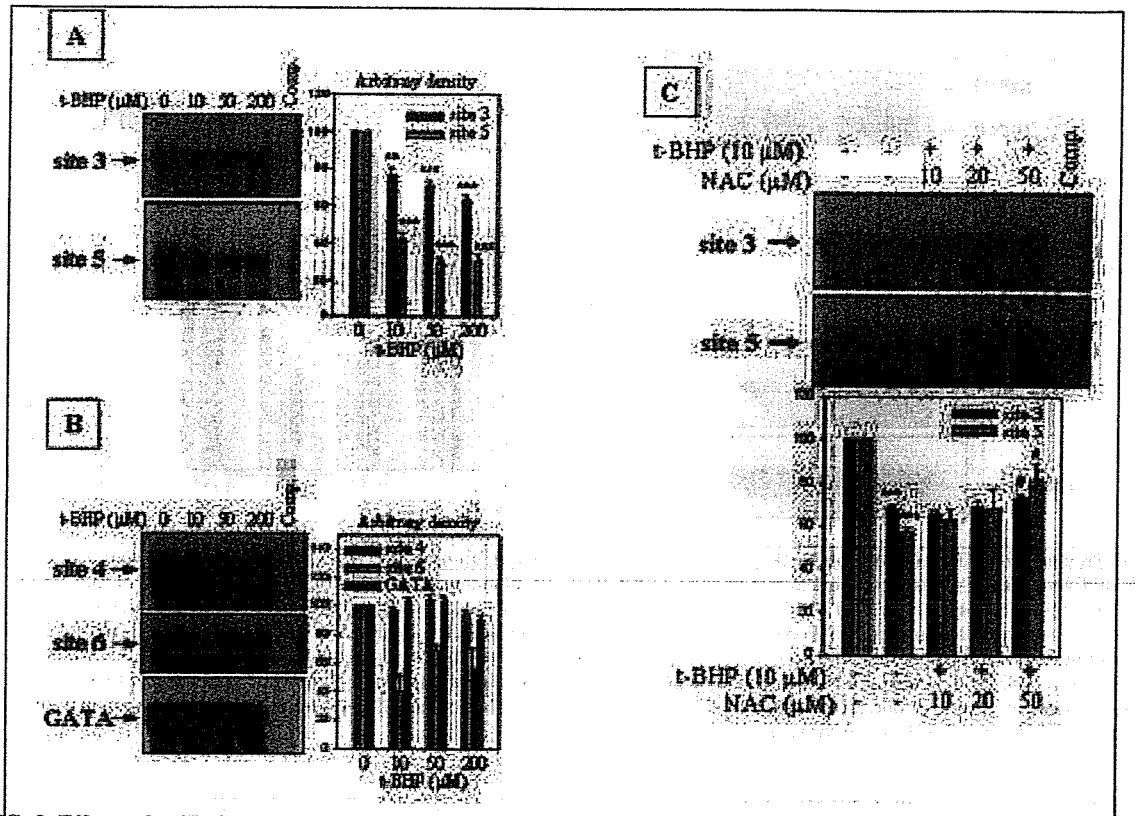

FIG. 3. Effects of oxidative stress on DNA binding activities of unidentified transcription factors *in vitro*. DNA binding activities are shown of liver nuclear protein (25 μg protein) inflicted by *t*-BHP (μ*M*) *in vitro*, using 6-month-old rats (**A**) and (**B**). An additional antioxidant, NAC (10 ~ 50 μ*M*) and oxidant, *t*-BHP (10 μ*M*) were co-treated to sites 3 and 5 (**C**). CTL, vehicle-treated control group; comp., competitive confirmation with content of excess unlabeled oligonucleotides (200-fold competitor). As statistical significance, results of one factor ANOVA: $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* *t*-BHP untreated group, and $\#p < 0.05$ versus *t*-BHP only treated group, respectively.

activities and aging, compared to activity at sites 3 and 5 indicates that the decreased DNA binding activity at sites 3 and 5 occurs in their promoter regions due to increased oxidative stress. To verify these results, we checked binding activities of the SMP30 promoter region utilizing oxidative stress-promoting, LPS-treated rats. Findings from the LPS treatment that elicits oxidative stress showed suppressed nuclear binding activity and that the antioxidant, NAC, blocked decreased DNA binding activities at sites 3 and 5, further supporting the binding reaction's sensitivity to the redox condition. However, the exact nature of oxidative modifications that influence the binding reaction is yet to be determined.

Redox-based gene expression has emerged as a fundamental regulatory mechanism of several redox-sensitive transcription factors that have been ascribed as apparent redox-sensing activity (24, 30). The functional thiol group of conserved cysteinyl residues in signal proteins account for their redox-sensing properties, which are oxidized and potentially affect redox signaling. Signals are transduced from the cell's surface into the nucleus through phosphorylation and dephosphorylation chain reactions of amino acid residues such as tyrosine and serine/threonine (33). Protein phosphorylation is one of the most fundamental mediators of cell signaling and is redox-sensitive. DNA-binding proteins, like NF-κB, AP-1, and p53, contain reactive thiols in their binding regions, and also are involved in the regulation of the redox status (26, 27). When the cysteine residues of a redox-sensitive transcription factor are phosphorylated, DNA binding activity is upregulated or downregulated by oxidative stress-induced phosphorylation (18).

Our previous work suggested that the downregulation of SMP30 with age is likely due to increased oxidative stress (19). In the current study, decreased SMP30 was further explored with the use of LPS and *t*-BHP challenges, both of which elicit oxidative stress that mimic what might occur with aging. Both *t*-BHP and LPS also are reported to be potent stimuli that induce oxidative stress in various cell culture systems, including hepatocytes, erythrocytes, and fibroblasts (5, 29), and several tissues such as brain, testis, and heart (2, 10). With *t*-BHP and LPS treatments, binding sites 3 and 5 showed significantly decreased binding activity, indicating the redox-sensitive nature of the DNA binding reaction. Results showing downregulated SMP30 by oxidative stress and the counteraction by CR strongly suggested that the change in SMP30 occurring during aging is likely elicited by oxidative stress, influencing the redox-responsive binding at sites 3 and 5.

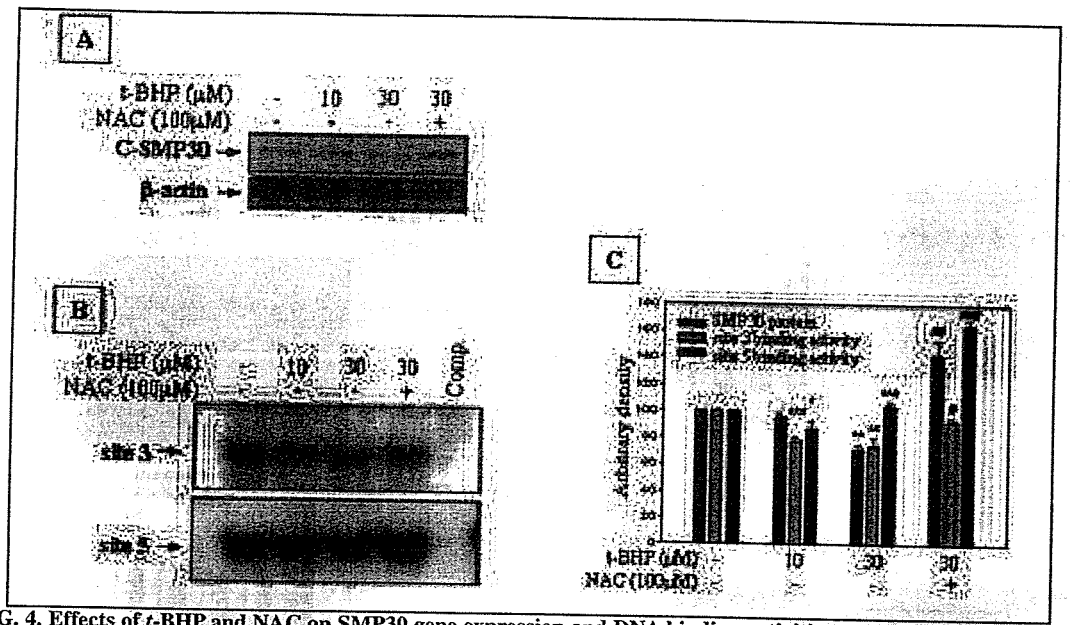

FIG. 4. Effects of *t*-BHP and NAC on SMP30 gene expression and DNA binding activities at binding sites 3 and 5 in cultured Ac2F liver cell. Cells were incubated with *t*-BHP and NAC simultaneously for 1 h or 5 h for fractionation of nuclear or cytoplasmic fraction, respectively. (**A**) SMP30 gene expression in cytosol (50 μg protein) was detected by Western blot. β-actin was used for an equal loading control. (**B**) Binding sites 3 and 5 were detected by EMSA with incubating ^{32}P-end-labeled oligonucleotides and nuclear protein extracts (15 μg protein). (**C**) Densitometric measurements showed effects of *t*-BHP and NAC in (**A**) and (**B**). C-SMP30, cytosolic SMP30; Comp., content of excess unlabeled oligonucleotides (200-fold competitor). As statistical significance, results of one factor ANOVA: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ versus *t*-BHP untreated group, and $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$ *vs.* *t*-BHP treated group, respectively.

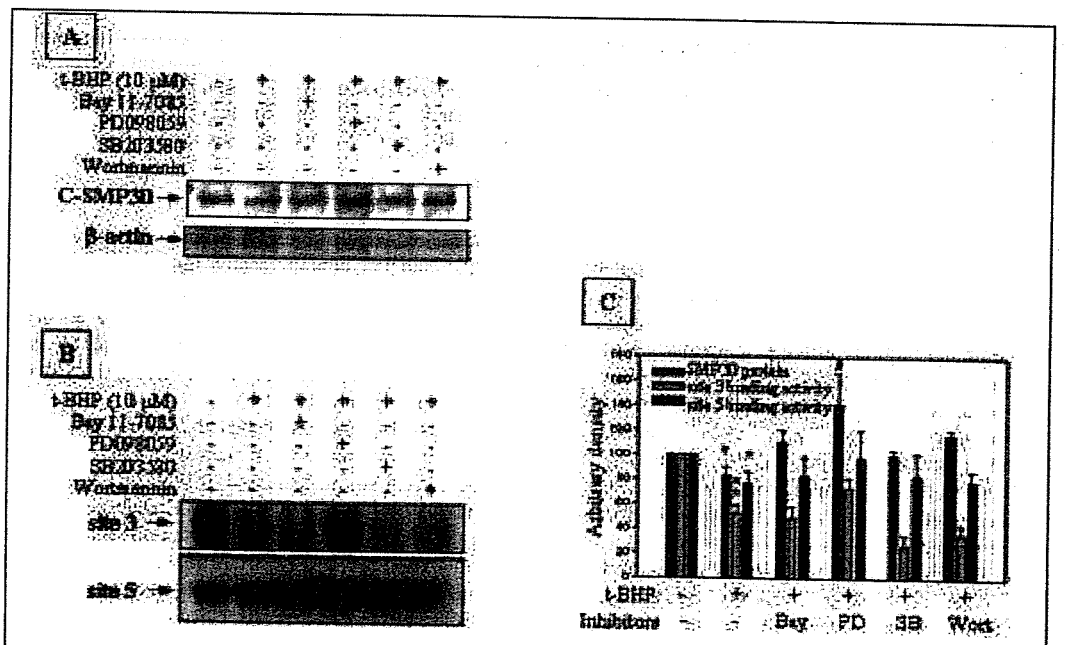

FIG. 5. Effects of PD098059 on SMP30 gene expression and DNA binding activities at binding sites 3 and 5 in cultured cell. Cells were incubated with kinase inhibitors and *t*-BHP simultaneously for 1 h or 5 h for fractionation of nuclear or cytoplasmic fraction, respectively. Bay 11–7085 (2 μ*M*), PD098059 (10 μ*M*), SB203580 (10 μ*M*), and Wortmannin (100 n*M*) were treated for inhibition of specific kinases. Kinase inhibitors were treated 10 min previously, and then *t*-BHP (10 μ*M*) were treated. (**A**) SMP30 gene expression in cytosol (50 μg protein) was detected by Western blot. β-Actin was used for an equal loading control. (**B**) Binding sites 3 and 5 were detected by EMSA with incubating ^{32}P-end-labeled oligonucleotides and nuclear protein extracts (15 μg protein). (**C**) Densitometric measurements showed effects of *t*-BHP and NAC in (**A**) and (**B**). C-SMP30, cytosolic SMP30; Comp., content of excess unlabeled oligonucleotides (200-fold competitor). As statistical significance, results of one factor ANOVA: $*p < 0.05$, $**p < 0.01$ versus *t*-BHP untreated group, and $\#p < 0.05$ versus *t*-BHP treated group, respectively.

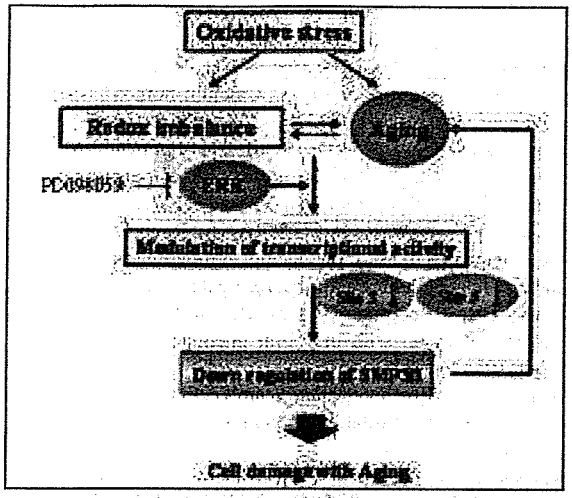

FIG. 6. Potential pathway for SMP30 gene expression regulation via ERK. Age-related oxidative stress leads to redox imbalance, decreasing SMP30 gene expression. Binding sites 3 and 5 that concern redox-sensitive SMP30 transcription are downregulated by oxidative stress and regulated by the ERK pathway. This is a hypothetical scheme for the age-associated decrease in SMP30 by oxidative stress that focuses on binding sites 3 and 5. SMP30 plays an important role as a Ca^{2+} regulating protein, which removes Ca^{2+} from the cytosol across the plasma membrane. In aged tissues, the gene expression of SMP30 is decreased, thereby influencing the alteration of the signaling system and the emergence of age-associated deterioration of cellular function associated with hypoxia, inflammation, and aging (6). *Thin arrow*, present work; *bold arrow*, reported results.

Given the ubiquitous expression of the MAPK pathway, the high degree of evolutionary conservation, and the wide range of cell-surface stimuli that trigger ERK activation, it is not surprising that this signaling module is involved in a vast number of cellular functions, including proliferation, differentiation, survival, migration, and adhesion. The large number of various stimuli that can lead to ERK activation renders ERK an essential signaling cross-road within the cell. Compelling evidence links ERK activation to cell degeneration and even cell death by exerting noxious effects leading to excitotoxicity or oxidative stress by increasing reactive oxygen species (ROS) (3). It was suggested that the kinetics and duration of ERK activation may determine whether downstream targets will trigger beneficial or detrimental effects on cells (e.g., a prolonged activation of ERK following ROS elevation). Additionally, cellular senescence is characterized by the ERK pathway in its participation in cell death due to oxidative stress and calcium dysregulation (34). Investigators report that senescent cells accompany the activation of the ERK pathway, which is consistent with reports that show a defective accumulation of ERK1/2 entered senescence after serial passage in the cell (3, 34).

To date, finding a link between the ERK pathway and SMP30 has not been reported. In this study, we showed that the inactivation of signal pathway ERK by the inhibitor, PD098059 may rescue the cell from the oxidative stress-induced decrease in SMP30 gene expression via binding sites 3 and 5. These molecular modulations revealed the decline of SMP30 gene expression through the regulation of the ERK pathway. Our study showed that the downregulated transcriptional expression of SMP30 and the downregulated SMP30 during aging are likely caused by decreased binding activity of transcription factors at sites 3 and 5 due to increased age-related oxidative stress. Our study further showed that the downregulation of both SMP30 gene expression and its transcriptional activity are implicated in the ERK pathway.

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ABBREVIATIONS

ANOVA, analysis of variance; bp, base pair; C-SMP30, cytosolic senescence marker protein-30; CR, calorie restriction; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal regulated kinase; GATA, transcription factor GATA binding protein; JNK, c-Jun-N-terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen activated protein kinase; NAC, *N*-acetylcysteine; NF-κB, nuclear factor κB; PVDF, polyvinylidene fluoride; ROS, reactive oxygen species; SMP30, senescence marker protein-30; Sp1, transcription factor stimulating protein, *t*-BHP, *tert*-butylhydroperoxide; TNFα, tumor necrosis factor α.

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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

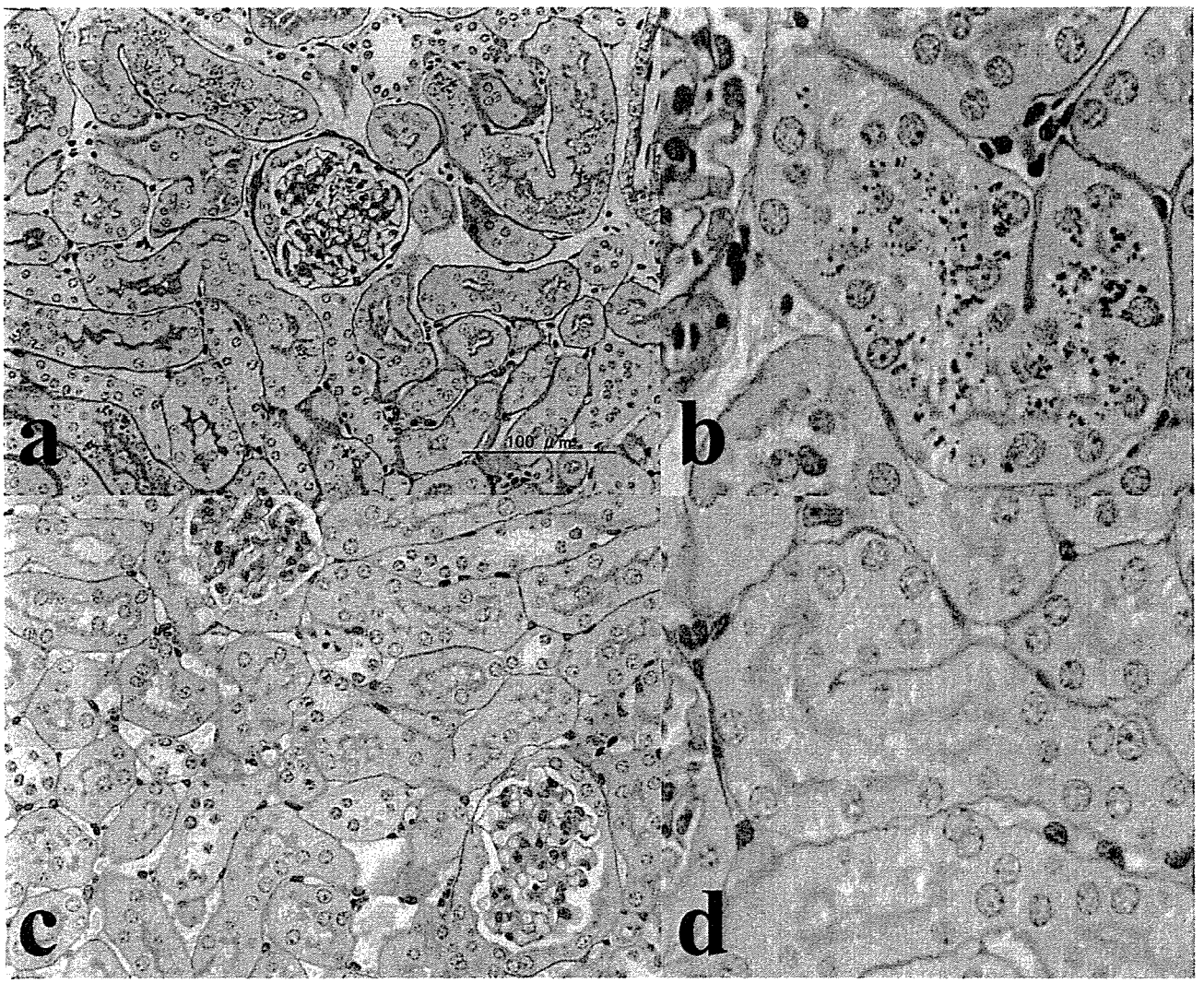

Fig. 1. Lipofuscin deposits accumulate abundantly in renal tubular epithelia of 12-month-old SMP30-KO mice. Paraffin-embedded tissue fixed in 10% formalin were cut at 2 μm and stained with periodic acid-Schiff for histopathological assessment. **a and b.** In the section from a 12-month-old SMP30-KO 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

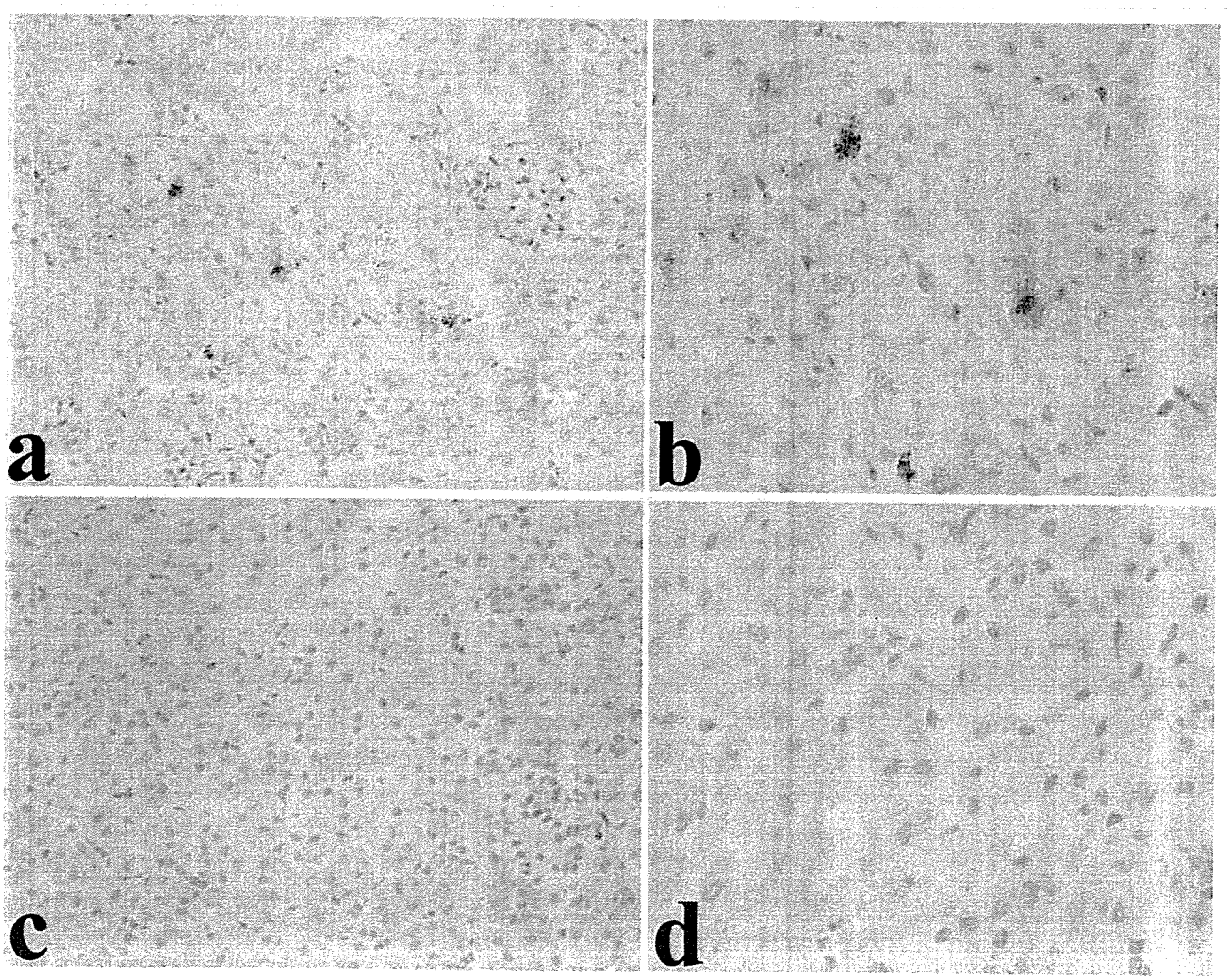

Fig. 2. a and b. SA-ß-GAL staining is positive in kidney tissue from 12-month-old SMP30-KO mice. SA-ß-GAL expression in the kidney section from a 12-month-old SMP30-KO mouse was detected only in proximal tubules, not glomeruli or vessels. **c and d.** Hematoxylin and eosin staining revealed lipofuscin 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

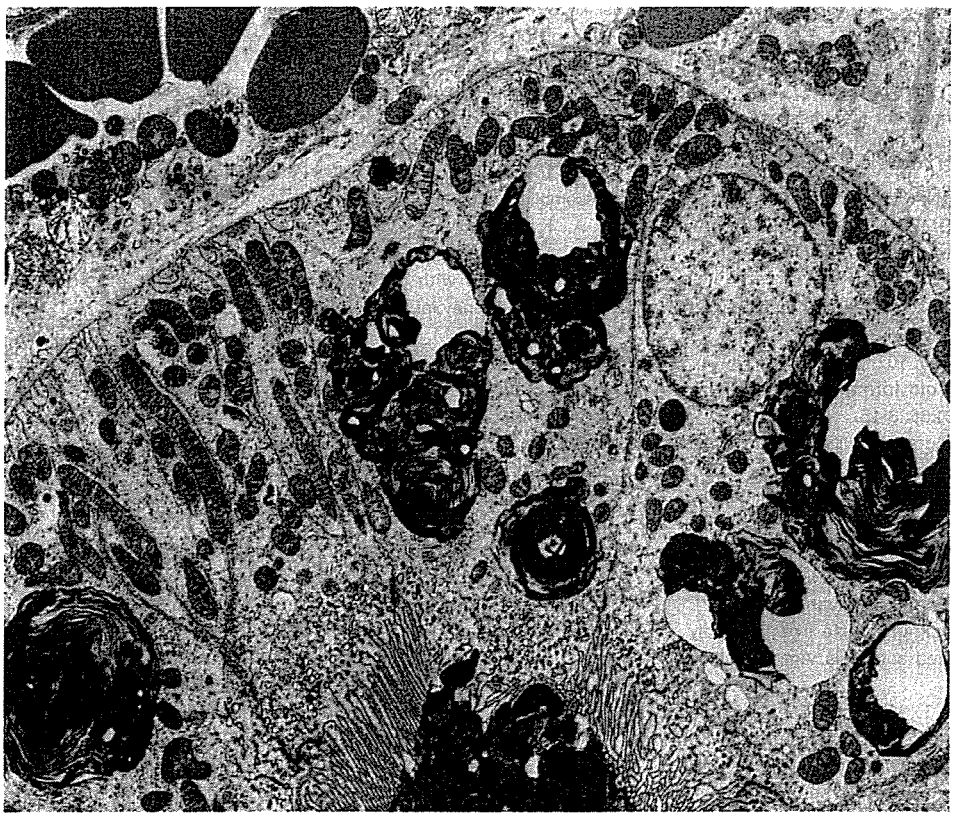

Fig. 3. Ultrastructural analysis revealed greatly enlarged lysosome containing an electron dense substance in a SMP30-KO mouse. x 800

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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MEETING REPORT

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The seventh Korea–Japan joint symposium on cancer and ageing research: molecular targets in cancer and ageing research

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Introduction

There is a significant association between advanced age and an increased incidence of cancer. It is thus imperative to discuss issues related to cancer and ageing together. The unique symposium, the seventh Korea–Japan joint symposium on cancer and ageing research: molecular targets in cancer and ageing research, was held from 3 to 4 November 2005 at a nice resort town named Muju in Korea. This seventh symposium sponsored by the Journal of Cancer Research and Clinical Oncology brought together two groups of scientists working in the fields of cancer and ageing biology. This article summarizes the findings of this symposium, which highlights the intimate relationship between carcinogenesis and ageing.

Plenary lectures, sessions, posters

Plenary lecture 1: Linkage between ageing and cancer through an endogenous cell death molecule, TIS21/BTG2/PC3

Dr. In Kyoung Lim (Ajou University) presented a fabulous lecture about TIS21/BTG2/PC3 molecule which belongs to anti-proliferative genes. Her recent data suggested that TIS21 regulates G1/S cell cycle both wild type p53-dependently and independently. TIS21 can also p53 independently induce G2/M arrest of U937 cells, resulting in cell death. Therefore, TIS21 was suggested as a pan-cell cycle regulator. TIS21 expression is highly active in mouse fetal liver, brain ventricular zone and spinal cord as well as mouse embryonal

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stem cells, indicating a potential role for TIS21 in neurogenesis and embryo development. However, the expression is lost in liver and neuronal tissue after birth suggesting a role for TIS21 in the ageing process. Furthermore, TIS21 expression is totally lost in renal cell tumors and prostate tumors in humans, whereas it is consistently expressed in the counterpart normal tissue. Finally, overexpression or knockout of TIS21 gene was found to induce the growth arrest or the abnormality of vertebral patterning during mice development, respectively. Therefore, her conclusion was that TIS21 was suggested as a regulator of cell cycle, thus acting as a tumor suppressor and also as a protein related with ageing phenotypes.

Plenary lecture 2: Exhaustion of hematopoietic stem cells by reactive oxygen species through p38MAPK

Development of stem cell research is essential for regenerative medicine and cancer therapeutics. Dr. Toshio Suda (Keio University) gave a plenary lecture on hematopoietic stem cells (HSCs). HSCs expressing the receptor tyrosine kinase Tie2 adhere to osteoblasts in the bone marrow (BM) niche. The interaction of Tie2 and its ligand Angiopoietin-1 (Ang-1) leads to tight adhesion of HSCs to stromal cells through N-cadherin, resulting in maintenance of long-term repopulating activity of HSCs. Thus, the Tie2/Ang-1 signaling pathway plays a critical role in maintaining HSCs in a quiescent state in the BM niche. As for the self-renewal capacity of HSCs in the niche, loss of "Ataxia telangiectasia mutated" (ATM) protein upregulates radical oxygen species (ROS) and the cyclin-dependent kinase inhibitor p16, which results in the defect in stem cell function. Treatment with anti-oxidative reagents restored the reconstitutive capacity of ATM-/- HSCs, indicating the important role of ATM in HSC function.

Session A: Chaired by Dr. Sataro Goto
(Toho University)

Four speakers gave their presentations on ageing, Alzheimer's disease, and oxidative stress-mediated apoptosis during this session. Dr. Tomonori Hayashi (Radiation Effects Research Foundation) described immunological status of healthy atomic (A)-bomb survivors after more than half a century of exposure. He reported that inflammatory markers such as IL-6, IL-10, TNF-α, IFN-γ and C-reactive protein, and immunoglobulins (IgG, IgA and IgM) levels were elevated with increased dose of the radiation. Comparing with age-related changes of these markers in unexposed normal population, he found that exposure to 1 Gy of radiation corresponds to about 9 years of ageing. He speculates that A-bomb irradiation might accelerate immunological ageing by promoting inflammation and antibody production.

Gamma-secretase is believed to play an important role in the pathogenesis of Alzheimer's disease. Dr. Inhee Mook-Jung (Seoul National University) threw light on the regulator of the gamma-secretase activity. Gamma-secretase is endogenously regulated via ERK1/2 dependent MAPK pathway. The inhibition of ERK1/2 activity, either by treatment with an MEK inhibitor or an ERK knock down transfection, dramatically increased gamma-secretase activity. Conversely, increased ERK1/2 significantly reduced gamma-secretase activity, demonstrating down-regulation of gamma-secretase activity by ERK1/2.

Dr. Naoaki Ishii (Tokai University School of Medicine), who had discovered mev-1 mutant in *Caenorhabditis elegans* with premature ageing phenotypes that have a defect in cytochrome b large subunit of the complex II in mitochondrial respiratory chain, established a transgenic mouse 3T3 cell line with a similar mutation in the homologous subunit. He found increased production of superoxide from the complex of the cells that leads to excessive apoptosis. Interestingly, a part of surviving cells after crisis due to apoptosis were transformed, forming tumor, apparently benign, upon injection into nude mice. He suggested that mitochondrial oxidative stress not only accelerates ageing but also causes apoptosis and cancer.

Dr. Naoki Maruyama (Tokyo Metropolitan Institute of Gerontology) reported that citrullination, a form of post-translational modification in protein, was found in pathological as well as physiological situations. Citrullin residues are formed by catalysis of peptidylarginine deaminase (PAD) from arginine residues. The modified proteins were found in large numbers in the hippocampus of patients of Alzheimer's disease and in Bowman's capsule of obstructive nephropathy patients. The increase was accompanied by an elevated expression of PAD type II. Citrullinated proteins were also found in normal differentiated epidermal cells, suggesting a physiological role of this modification.

Session B: Chaired by Dr. Yasuhito Yuasa
(Tokyo Medical and Dental University)

In this session, the mechanisms underlying senescence and carcinogenesis were presented by four speakers. Dr. Jeong-Soo Park (Dankook University) highlighted increased caveolin-1 in senescent human mesenchymal stem cells (MSCs). Bone marrow-derived human MSCs divided poorly and took flat and enlarged morphology after expanded in culture over a certain number of cell passages, which resembled characteristic features of senescent cells. Adipogenic differentiation potential also sharply declined as they approached the end of their proliferative life span. When cavelolin-1 was overexpressed in young MSCs, adipogenic differentiation was significantly suppressed, indicating that loss of adipogenic differentiation potential in senescent MSCs is mediated by the overexpression of caveolin-1.

Dr. Eisaburo Sueoka (Saga University) described the inhibition of DNA repair capacity by heterogeneous nuclear ribonucleoprotein B1 (hnRNP B1) as a pivotal role in lymphomagenesis and lung carcinogenesis. hnRNP B1 levels in normal CD4 positive T lymphocytes are negligible, but they increase along with disease progression in ATL patients. hnRNP B1 interacted with DNA-dependent protein kinase (DNA-PK) complex, and recombinant hnRNP B1 protein dose-dependently inhibited DNA-PK activity. He assumes that overexpression of hnRNP B1 occurring in the early stage of carcinogenesis inhibits DNA-PK activity, resulting in subsequent accumulation of erroneous rejoining of DNA double-strand breaks, causing tumor progression.

Dr. Young-Joon Surh (Seoul National University) discussed redox sensitive transcription factors as prime molecular targets for chemoprevention and cytoprotection. Components of the cell signaling network, especially those that converge on redox-sensitive transcription factors including nuclear factor-kappaB and AP-1 involved in mediating inflammatory response, have been implicated in carcinogenesis. Modulation of cellular signaling involved in chronic inflammatory response by anti-inflammatory agents, therefore, provides a rational and pragmatic strategy in molecular target-based chemoprevention. Induction of phase-2 detoxifying or antioxidant enzymes represents an important cellular defense response to oxidative and electrophilic insults. Nrf2, another redox-sensitive transcription factor, plays a crucial role in regulating phase-2 detoxifying/antioxidant gene induction. Many chemopreventive and chemoprotective agents have been found to activate this particular transcription factor, thereby potentiating cellular antioxidant capacity.

Telomeres are shortened in cellular ageing in vitro as well as in cells in vivo with advancing age. Dr. Makoto Kammori (University of Tokyo and Tokyo Metropolitan Institute of Gerontology) has developed quantitative fluorescence in situ hybridization (Q-FISH) that allows one to study telomere changes in specific chromosomes. Using Q-FISH technique, he discovered that telomere shortening in inactive X chromosomes (Xq) was accelerated with ageing in vitro in human female fibroblasts (TIG-1), creating telomeric association with another chromosome with shortened telomeres. His findings suggest that short telomere on the chromosome with inactive Xq contributes to loss of the inactive Xq alleles frequently observed in female cancers.

Session C: Chaired by Dr. Gou Young Koh (Korea Advanced Institute of Science and Technology)

Dr. Yuji Yamanashi (Tokyo Medical and Dental University) reported on roles of the Dok-family adaptors in hematopoietic cells. Dok-1 and Dok-2 are closely related rasGAP-associated adaptors that are expressed preferentially in hematopoietic cells. Protein tyrosine kinases (PTKs) are involved in TLR4-mediated lipopolysaccharide (LPS) signaling and Dok-family adaptors were tyrosine phosphorylated after LPS treatment of macrophages. This suggests that Dok-1 and Dok-2 are primarily responsible for controlling the rapid process of LPS signaling, to which inducible regulators cannot react.

Eui-Ju Yeo (Gachon Medical School) described molecular mechanisms underlying the age-dependent cAMP profiles in human diploid fibroblasts (HDFs) stimulated by lysophosphatidic acid (LPA). In senescent cells, LPA-dependent Gi activation was reduced, with a consequent reduction in Gi-suppressed cAMP levels, without alterations in the levels of Gi proteins. However, in young cells, when Gi activity was inhibited by pertussis toxin pretreatment, or when its expression was blocked by siRNA, the pattern of changes in cAMP levels in response to LPA was similar to that seen in senescent cells. Together with other data, she suggested that LPA-dependent increase of cAMP level in senescent HDFs is associated with reduced Gi, increased level of PKC-dependent A-kinase anchoring proteins and adenylyl cyclases, and PKC-dependent stimulation of their activities through direct or indirect interactions, and provide an explanation for the age-dependent differences in cAMP-related physiological responses.

Endothelial cells in atherosclerotic lesions have shorter telomeres and, therefore, have reduced life span. Dr. Youji Mitsui (Tokushima Bunri University) has established immortal human endothelial cell lines that may be used for cell therapy of atherosclerosis. He introduced human telomere reverse transcriptase gene into human umbilical vein endothelial cells. The transgenic cells formed capillary like structures in the presence of VEGF or FGF-2. No tumor was developed in nude mice on injection of the cells, proving that immortal cells retain normal properties. Implantation of the cells in balloon-injured carotid aorta in nude rats gave promising morphological results in the repair of the vascular wall.

Dr. Hong-Duk Youn (Seoul National University) discussed p53 stabilization and transactivation by a von Hippel–Lindau (VHL) protein. VHL disease is a rare autosomal dominant cancer syndrome. Hypoxic inducible factor alpha (HIFalpha) is a well-known substrate of a von Hippel–Lindau tumor suppressor protein (pVHL). He found that pVHL directly associated with and stabilized p53 by suppressing the mdm2-mediated ubiquitination and nuclear export of p53, and this result suggested that the tumor suppressor pVHL has an unexpected function to upregulate another tumor suppressor p53.

Session D: Chaired by Dr. Young-Joon Surh (Seoul National University)

Dr. Toru Suzuki (University of Tokyo) reported on a novel biological function of anti-proliferative protein Tob. Detection of apoptosis-related DNA fragmentation by TUNEL method indicated that Tob-overexpressed cells were highly resistant to UV-irradiation. Conversely,

RNA interference-mediated Tob suppression led cells to be more sensitive to UV-irradiation. Moreover, apoptotic cells were increased in tob-deficient mouse embryonic fibroblasts as compared with those in wild-type fibroblasts upon UV-irradiation. These data indicate that Tob is a physiological antiapoptotic molecule.

Dr. Yun-Sil Lee (Korea Institute of Radiological and Medical Sciences) described the possibility of heat shock protein 25/27 as a molecular target for cancer prediction. She indicated that HSP25/27 directly bound PKCdelta, which showed radiation- or oxidative stress-induced apoptosis, and inhibited PKCdelta-mediated apoptosis. HSP25/27 was overexpressed in lung cancer tissue, which showed resistance to radiation or chemotherapeutic agents, suggesting that HSP25/27 acts as a predictive factor of resistance to radiation or chemotherapeutic agents.

Dr. Hisataka Moriwaki (Gifu University) reported on strategy and mechanism for prevention of liver cancer through retinoid analysis. Retinoid is a collective term for vitamin A analog that binds to nuclear retinoid receptors, i.e., retinoic acid receptors and retinoid X receptors (RXRs). After ligand coupling, these receptors form as dimer, bind to response element of the gene, and regulate the gene expression as a transcriptional factor. Biological phenotypes of transcriptional regulation by retinoid include cellular differentiation and tissue morphogenesis and apoptosis. Dr. Moriwaki performed clinical trials of retinoid analog to inhibit second primary hepatomas. Supposed molecular mechanism of the action of the compound and aberrant metabolism of RXR and its role in liver carcinogenesis were reviewed.

Dr. In-Gyu Kim (Seoul National University) highlighted differential alternative splicing of transglutaminase 4 (Tgase 4) in prostate cancer and benign prostate hyperplasia. Tgase 4 is expressed mainly in prostate and secreted into seminal fluid. He examined the expression of Tgase 4 in human prostate, and found that Tgase 4 is alternatively spliced in exon 2, a splicing donor site of intron 1 and exon 4, generating 4 different mRNAs: L, M1, M2 and S forms. Of the 80 benign prostate hyperplasic tissues, all three forms were expressed in 45, whereas M and S forms were expressed in 35. By contrast, 41 out of 48 prostate cancer tissues express only M and S forms. Thus, the pattern of alternative splicing of the Tgase 4 gene in prostate tissues is very different from that in prostate cancer, indicating the altered regulation of alternative splicing in prostate cancer.

Session E: Chaired by Dr. Yuji Yamanashi (Tokyo Medical and Dental University)

Dr. Yoshimitsu Akiyama (Tokyo Medical and Dental University) demonstrated frequent epigenetic silencing of bone morphogenetic protein 2 (BMP-2) gene through promoter hypermethylation in gastric carcinomas. BMP-2 mRNA was found to be activated after 5-aza-2′-deoxycytidine treatment of the methylation-positive cancer cells. Immunohistochemical staining revealed a significant association between BMP-2 methylation and loss of its protein expression. BMP-2 methylation was detected in 24 of the 56 (42.9%) primary cases, and more often observed in diffuse type than in intestinal type gastric carcinomas. Thus, aberrant BMP-2 methylation and the resultant loss of BMP-2 expression may be related to gastric carcinogenesis, particularly in the diffuse type.

Dr. Takashi Kuzuhara (Tokushima Bunri University) investigated the structure–function relationship of *Helicobacter pylori* (*H. pylori*) TNF-inducing protein (Tip) and Gram-positive bacterial penicillin binding protein identified by Psi-Blast search. Tip has already been reported to have carcinogenic potential as a tumor promoter. These two genes conserved the motif-like structures, and phylogenic tree analysis indicated that Tip was closer to the penicillin binding proteins of Gram-positive bacteria, based on their primary structures, than those of *H. pylori*. These data suggest that Tip and penicillin binding protein are derived from a common ancestral protein, and that the Tip gene may be transferred horizontally from Gram-positive bacteria to *H. pylori*. This work also provides evidence that horizontal transfer of bacterial genes is involved in human carcinogenesis.

Recently, therapeutic antibodies targeting the tumor-associated antigens have become clinically available. Anti-tumor associated antibodies are actively developed via multiple steps. Dr. Junho Chung (Seoul National University) overviewed several strategies of therapeutic antibody development. He devised a novel high-throughput generation and screening procedures for anti-tumor associated antigen rabbit/human chimeric antibodies. The efficacy of the selected antibodies in treatment of cancer is now being tested.

Dr. Midori Suenaga (Tokushima Bunri University) reported on enhancement of cancer prevention by multiple treatments with green tea catechins, such as EGCG, ECG, EGC and EC, in A549 human lung cancer cells. Four treatments with EGCG, EGC and ECG enhanced growth inhibition of A549 cells compared with a single treatment. Moreover, A549 cells with multiple treatments of catechins up-regulated GADD153, an apoptosis-related gene, and p21/WAF1 expression, suggesting that multiple administrations of green tea catechins enhance induction of apoptosis and cell cycle arrest in the cells. The synergistic enhancement by multiple administrations of green tea catechins suggests that the more green tea one drinks, the more protection against cancer one will have.

Poster presentations by young scientists and students

In addition to the regular sessions, 21 poster presentations on a wide spectrum of researches covering cancer and ageing were given by young scientists and students. The enthusiasm of the young participants in the presentations and discussions made the symposium a great success. The future for the symposium also holds great promise.