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Ascorbic acid deficiency accelerates aging of hepatic stellate cells with up-regulation of PPARγ

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Summary. Senescent cells have been observed in certain aged or damaged tissues. However, the information about the effects of aging on liver cells is limited. In the present study, we have examined age-related histological changes in the livers of senescence marker protein knockout ($SMP30^{-/-}$) mice, which are considered as a murine aging model due to the more sensitive response to apoptotic reagents and due to their shorter life span. In livers of old $SMP30^{-/-}$ mice, numerous hepatic stellate cells (HSCs) were hypertrophic and contained abundant microvesicular lipid droplets in cytoplasm. We have found that the expression of peroxisome proliferators-activated receptor γ (PPARγ), which is a protein related to lipid metabolism and HSC quiescence, was increased in hypertrophic HSCs by aging and vitamin C (VC) deficiency, whereas these phenomena were dramatically reduced by antioxidant treatment. Therefore, these prominent phenotypic changes can be considered as aging markers in the livers of animals which are subjected to antioxidant property evaluation.

Key words: SMP30 knockout mice, Aging, Ascorbic acid, PPARγ, Hepatic stellate cells, Hypervitaminosis A

Introduction

The antioxidant vitamin C (VC) plays an important role in maintaining body homeostasis. Various forms of cellular damage or oxidant stress can accelerate senescence in many cell types (Campisi and d'Adda di Fagagna, 2007). The study of the physiological aging processes of vital organs is important for understanding the functions of organisms in response to various stresses mediated by exogenous and endogenous stimuli (Vollmar et al., 2002). Senescent cells have been observed in certain aged or damaged tissues. However, there is very little information about senescent cells in the liver. Basically, the aging liver has been considered as a tissue with minor structural or biochemical changes (Propper, 1986; Vollmar et al., 2002; Ito et al., 2007).

VC is a reducing agent and antioxidant that plays the role of a free-radical scavenger (Buettner and Jurkiewicz, 1993). VC is synthesized in the liver of most mammalian species. However, some species, such as primates and guinea pigs, are unable to synthesize VC by mutation of L-gulonolactone oxidase (GLO) (Nishikimi et al., 1994). It has been shown that senescence marker protein 30 knockout ($SMP30^{-/-}$) mice develop VC deficient conditions. SMP30 is a 34kDa protein that is mainly expressed in the liver, kidney, and lung. It is decreased with aging in an androgen-independent manner (Mori et al., 2004). SMP30 has been considered as a multifunctional protein providing protection to cellular functions from oxidative stress in various organs (Sato et al., 2006; Son et al., 2006; Park et al., 2008). SMP30 was demonstrated to be a unique gluconolactonase (GNL), which is involved in VC biosynthesis (Kondo et al., 2006). $SMP30^{-/-}$ mice fed a VC deficient diet displayed symptoms of scurvy and accelerated aging, as compared to the wild type mice (Ishigami et al., 2004; Kondo et al., 2006). Therefore, the $SMP30^{-/-}$ mouse has been regarded as a good experimental model to examine the aging process by oxidant stress (Maruyama et al., 2004; Kashio et al., 2009). A previous study has shown some age-related histological changes on the liver induced by VC deficiency in $SMP30^{-/-}$ mice (Ishigami et al., 2004). This study showed an increase of lipid, abnormal

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mitochondria, and enlarged lysosomes in aging hepatocytes of SMP30$^{-/-}$ mice compared to age-matched wild-type mice (Ishigami et al., 2004). In addition, we observed numerous numbers of hypertrophic HSCs in the aging liver of SMP30$^{-/-}$ mice. The number of hypertrophic HSCs was dramatically reduced by antioxidant treatment. Subsequently, this histological change is determined as relating to VC, lipid, and PPARγ metabolisms in the aging liver. Therefore, we suggest that these prominent phenotypic changes are new redox or aging markers of the liver.

Materials and methods

SMP30 knockout (SMP30$^{-/-}$) mice

SMP30$^{-/-}$ mice were kindly provided by Dr. Ishigami. These mice were generated by gene targeting as described previously (Ishigami et al., 2002). Genomic DNA of the SMP30$^{-/-}$ mice was purified from mouse tail tissue using a combination of several procedures as described (Henneberger et al., 2000). Primers for genotype analysis through PCR were described in our previous study (Park et al., 2008). Mice were maintained at 12h day/dark cycles in a controlled environment (22±2°C, relative humidity 50±10%) and fed *ad libitum* with standard mouse chow. Animal experiments were performed in accordance with the National Institute of Health (NIH) guidelines for the care and use of laboratory animals.

Examination of senescent changes in aging liver

All female SMP30$^{-/-}$ mice were fed VC (Sigma Aldrich, USA) with drinking water and determination of VC dose was performed as described (Kondo et al., 2006). Mice were divided into four groups. Group 1 was composed of 12-week-old SMP30$^{-/-}$ mice fed VC. Group 2 was composed of 12-week-old SMP30$^{-/-}$ mice not fed VC. Group 3 was composed of 32-week-old SMP30$^{-/-}$ mice fed VC. Group 4 was composed of 32-week-old SMP30$^{-/-}$ mice not fed VC. All mice were euthanized at 28 and 48 weeks old, respectively after 16 weeks.

Examination of antioxidant effect in aging liver

46 week-old male mice of SMP30$^{-/-}$ were used as experimental groups of old mice and 18 week-old male SMP30$^{-/-}$ mice were used as a young control group. All male SMP30$^{-/-}$ mice were fed VC with drinking water before the experiment beginning at birth, and then the mice were divided into four groups. The control group was Group1 that was composed of 18-week-old SMP30$^{-/-}$ mice fed VC drinking water. Group 2 was composed of 46-week-old SMP30$^{-/-}$ mice not fed VC. Group 3 and 4 were composed of 46-week-old SMP30$^{-/-}$ mice not fed VC and then given drinking water with 5% and 10% ENA actimineral Resource A (ENA-A), which has an antioxidant function (Hong et al., 2011), respectively. All mice were euthanized at 36 and 64 weeks old, respectively after 18 weeks.

Quantitative Senescence-Associated (SA) ß-galatosidase (ß-gal) assay

A quantitative assay for SA-ß-gal activity was performed according to methods by Castro et al. (2003). Briefly, tissue lysates were prepared as described in immunoblot analysis. Fifty micrograms of total protein were diluted with RIPA buffer to a final volume of 50 ul and placed in a 96 well microtiter plate. 100 ul of SA-ß-gal stain solution (pH 6.0) were added. The SA-ß-gal solution contained 1mg/ml 5-bromo-4-chloro-3-indolyl ß-D-galactoside (X-gal), 40 mM citric acid/sodium phosphate, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 2mM $MgCl_2$. The plate was then incubated at 37°C for 24h and the optical density (OD) was read at 590nm. pH 7.4 SA-ß-gal stain solution was used as a control.

Histology and immunohistochemistry (IHC)

Mice were anesthetized and then rapidly exsanguinated through the caudal vena cava. Liver pieces were quickly removed and fixed in 10% neutral buffered formalin, processed routinely and embedded in paraffin wax for hematoxylin and eosin (H&E) stain. For specific lipid staining, formalin fixed mouse livers were embedded in OCT compound and frozen in liquid nitrogen. The cryosections were stained with Oil red-O stain to visualize neutral lipids. The stained sections were then analyzed by light microscopy. For IHC, endogenous peroxidase activity was inhibited using 3% hydrogen peroxide and executed with a microwave antigen retrieval process in 10 mmol/L citrate. Monoclonal anti-desmin (Dako, Denmark), anti-α-SMA (Sigma Aldrich, USA) and polyclonal anti-PPARγ (Upstate, USA) were used for immunostaining. The antigen-antibody complex was visualized by an avidin-biotin peroxidase complex solution using an ABC kit (Vector Laboratories, USA). Sections were developed with 3,3'-diaminobenzidine and finally counter-stained with Mayer's hematoxylin.

Immunoblot analysis

Snap-frozen liver tissues were homogenized in RIPA buffer containing 1 mM Na_3Vo_4, 50 mM NaF, and protease inhibitor cocktail tablets (Roche, Germany). The soluble cytosolic protein was obtained from the lysate and protein concentrations were determined by the Bradford method. Protein samples were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). For immunoblotting, proteins were electro-transferred to a PVDF membrane (Schleicher & Schuell, Germany) and blocked with 3% bovine serum albumin in Tris-buffered saline (TBS). The membrane was immuno-blotted with rabbit polyclonal antibodies against PPARα

(Santa Cruz Biotechnology, USA), PPARγ (Upstate, USA). Monoclonal anti-ß-tubulin (Sigma Aldrich, USA) was used as a loading control. The primary antibodies were detected using horseradish peroxidase (HRP)-conjugated anti-rabbit or mouse IgG. Specific binding was detected using the Super Signal West Dura Extended Duration Substrate (Pierce, USA) and exposure to medical X-ray film (Kodak, Japan).

Statistical analysis

The results are presented as means ± SD. Statistical analyses were performed using the Student's *t* test. Statistical significance was assumed when $p<0.01$.

Results

VC deficiency accelerates cellular senescence of the liver

To examine whether VC deficiency increases cellular senescence, a quantitative SA-ß-gal assay was performed in the liver tissue (Fig. 1). It has been shown that SA-ß-gal accumulates in senescent cells and that the SA-ß-gal assay is widely used as a biomarker of cellular senescence (Dimiri et al., 1995; Castro et al., 2003; Krizhanovsky et al., 2008). The level of SA-ß-gal activity at pH 6.0 was the lowest in 28-week-old mice fed with VC (Group 1), whereas it was the highest in 48-week-old mice without VC feeding (Group 4). At the same age of the mice, VC deficiency increase SA-ß-gal activity in the liver. Control experiments with SA-ß-gal stain solution at pH 7.4 showed almost no detectable change in OD at 590nm. This study demonstrated that the senescence of the liver was accelerated by VC deficiency.

Aging induces hypertrophy of HSCs which is associated with accumulation of lipid droplets

To examine the histological changes in the aging liver, we compared the liver morphology of young and old SMP30$^{-/-}$ mice. In microscopic examinations, numerous HSCs in old SMP30$^{-/-}$ mice were hypertrophic with abundant lipid droplets in the cytoplasms (Fig. 2A). Lipid droplets were observed as clear vacuoles in the cytoplasm. The nucleus of hypertrophic HSCs was stellate in appearance and usually located eccentrically by lipid droplets. Lipid droplets in HSCs were determined with red color by Oil red-O staining (Fig. 2B). Only a few HSCs were hypertrophic in 28-week-old mice fed with VC (Group 1), whereas the number of hypertrophic HSCs was increased when mice were getting older with VC deficiency. Group 4 (48-week-old mice without VC feeding for 16 weeks) had the greatest number of hypertrophic HSCs. Desmin is a marker for both quiescent and activated HSCs, while α-SMA is expressed only in activated HSCs (Senoo et al., 2007). Hypertrophic HSCs, as well as flattened HSCs at the lining of sinusoids, were positively stained with desmin in all groups, whereas α-SMA was normally expressed in smooth muscle cells around vessels, but not in HSCs (Fig. 2C). Thus, these data show that lipid accumulation is increased in HSCs by aging and that these HSCs are in a quiescent state.

PPARγ expression is increased in aging livers

We have previously determined that numerous HSCs become hypertrophic by aging. This morphological change of HSCs has been found in the liver with hypervitaminosis A (Kanel and Korula, 2005). It has been indicated that vitamin A (VA) increases with the lipids in the HSCs by aging. The peroxisome proliferators-activated receptors (PPARs) are ligand dependent transcription factors that belong to the nuclear hormone receptor family (Auwerx, 1999). These proteins play a major role in lipid metabolism and in HSC inactivation (Auwerx et al., 2003; Hazra et al., 2004; She et al., 2005). Three major subtypes of PPARs have been identified including PPARα, PPARß/δ and PPARγ. Two members, PPARα and PPARγ, are expressed in the liver (Rizzo and Fiorucci, 2006). Therefore, we examined the expression of PPARα and PPARγ in the livers from each group. There was no significant change in the expression of PPARα; however, the expression of PPARγ was increased by VC deficiency and aging (Fig. 3A,B). Although the age of animals was the same, PPARγ was expressed to a high level in VC deficient mice. The oldest mice without VC feeding (Group 4), showed the highest level of PPARγ expression in the liver. PPARγ-positive cells were mainly detected along the sinosoids that were co-

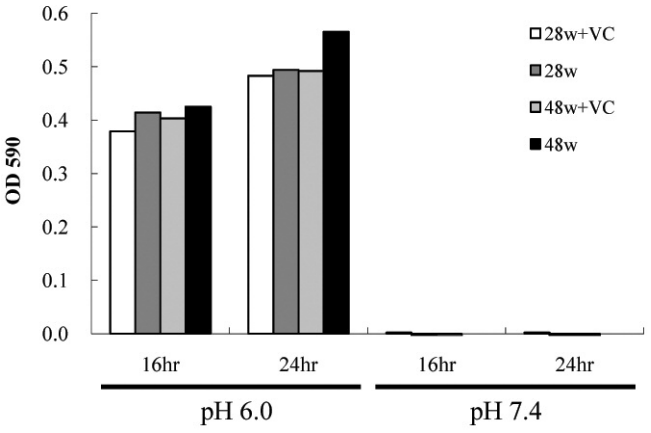

Fig. 1. SA-ß-gal activity is increased by aging and VC deficiency in the liver. SA-ß-gal activity was determined by quantitative assay using 50 μg of extract proteins from liver tissue as described in Materials and Methods.

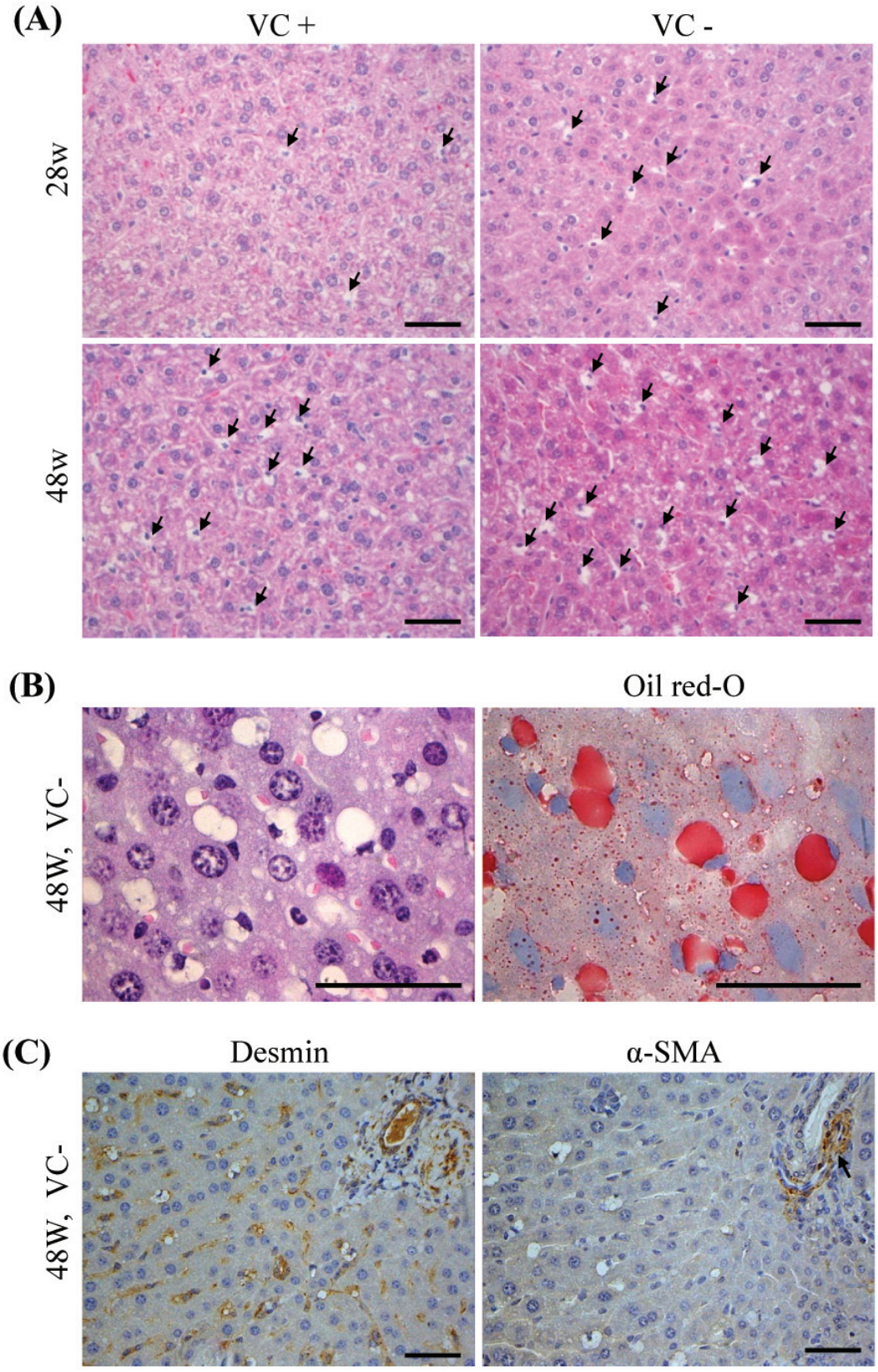

Fig. 2. HSCs show morphological changes by aging. **A.** HSCs (arrow) were hypertrophic with abundant lipid droplets in the cytoplasm H&E. **B.** Lipid droplets were observed as clear vacuoles in the cytoplasm, and the stellate nucleus was usually located eccentrically by lipid droplets in hypertrophic HSCs. Lipid droplets were stained red through Oil red-O staining. Oil red-O stain. **C.** Hypertrophic HSCs, as well as flattened HSCs in perisinusoidal regions, were positively stained by desmin, but not by α-SMA. α-SMA is normally expressed in smooth muscle cells around a hepatic artery (arrow). IHC of desmin and α-SMA. Scale bar: 50 μm.

localized with desmin-positive cells, but not with cells expressing α-SMA. Images with high magnification showed that PPARγ localization was mainly in HSCs, although some signals were occasionally observed in the hepatocytes (Fig. 3C). These studies revealed that hypertrophic HSCs containing lipid droplets are associated with an increased expression of PPARγ in the aging liver.

Antioxidant treatment decreases PPARγ expression and the number of hypertrophic HSCs in aging livers

We showed that the number of hypertrophic HSCs expressing PPARγ was increased by aging. Therefore, we asked if these changes might be corrected by antioxidant treatment. We used ENA-A as an antioxidant. ENA-A is an alkaline mineral water, which

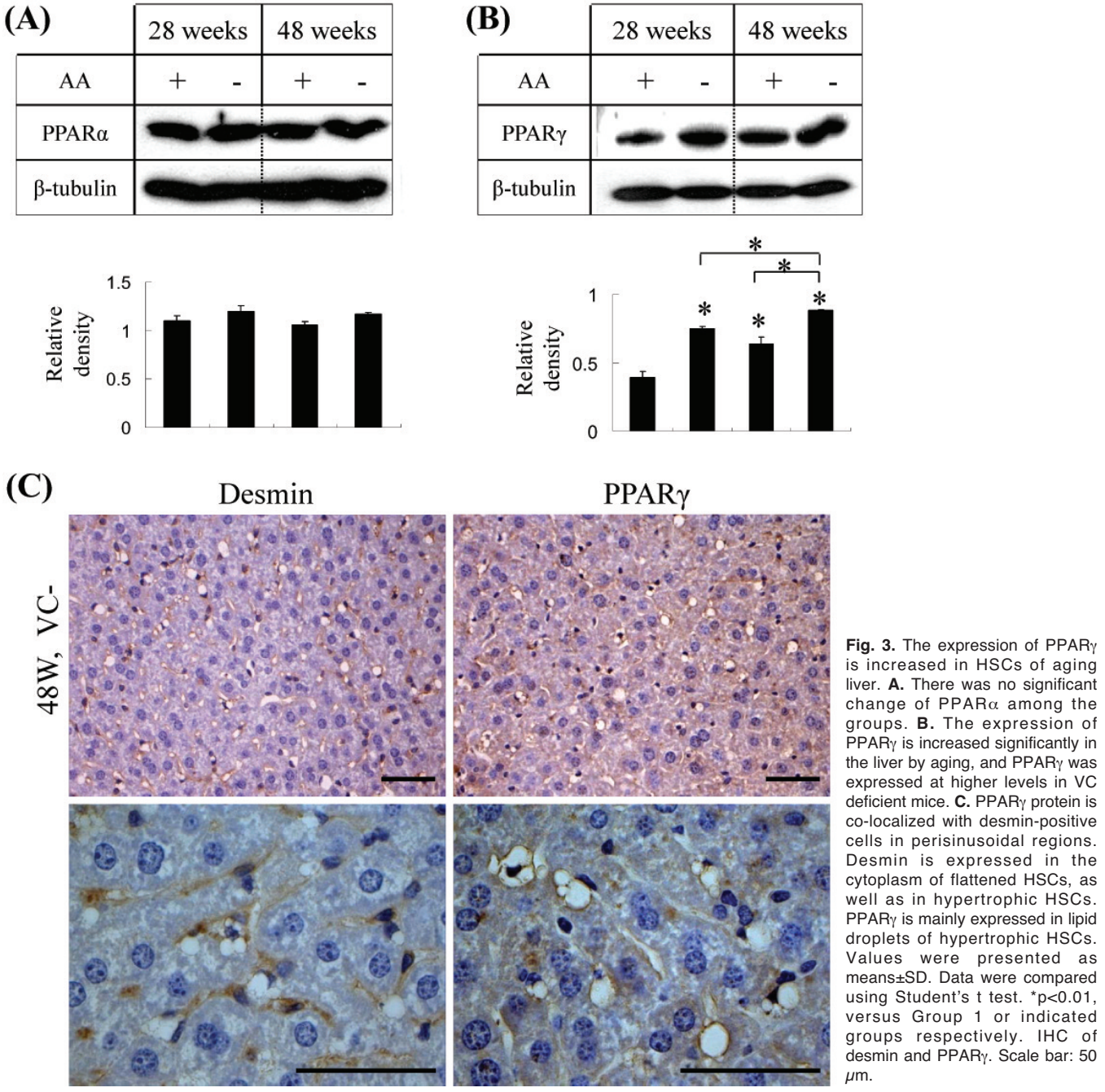

Fig. 3. The expression of PPARγ is increased in HSCs of aging liver. **A.** There was no significant change of PPARα among the groups. **B.** The expression of PPARγ is increased significantly in the liver by aging, and PPARγ was expressed at higher levels in VC deficient mice. **C.** PPARγ protein is co-localized with desmin-positive cells in perisinusoidal regions. Desmin is expressed in the cytoplasm of flattened HSCs, as well as in hypertrophic HSCs. PPARγ is mainly expressed in lipid droplets of hypertrophic HSCs. Values were presented as means±SD. Data were compared using Student's t test. *p<0.01, versus Group 1 or indicated groups respectively. IHC of desmin and PPARγ. Scale bar: 50 μm.

displays its hepatoprotective effect to CCl_4-induced liver injury through the antioxidant function (Hong et al., 2011). For this experiment, we used male SMP30$^{-/-}$ mice, in which we detected numerous hypertrophic HSCs. Thus, this lesion is sex-independent as SMP30 decreases with aging in an androgen-independent manner. In microscopic examination (Fig. 4A), only a few HSCs were hypertrophic in 36-week-old mice fed with VC (Group 1); however, the number of hypertrophic HSCs was increased to the highest level in

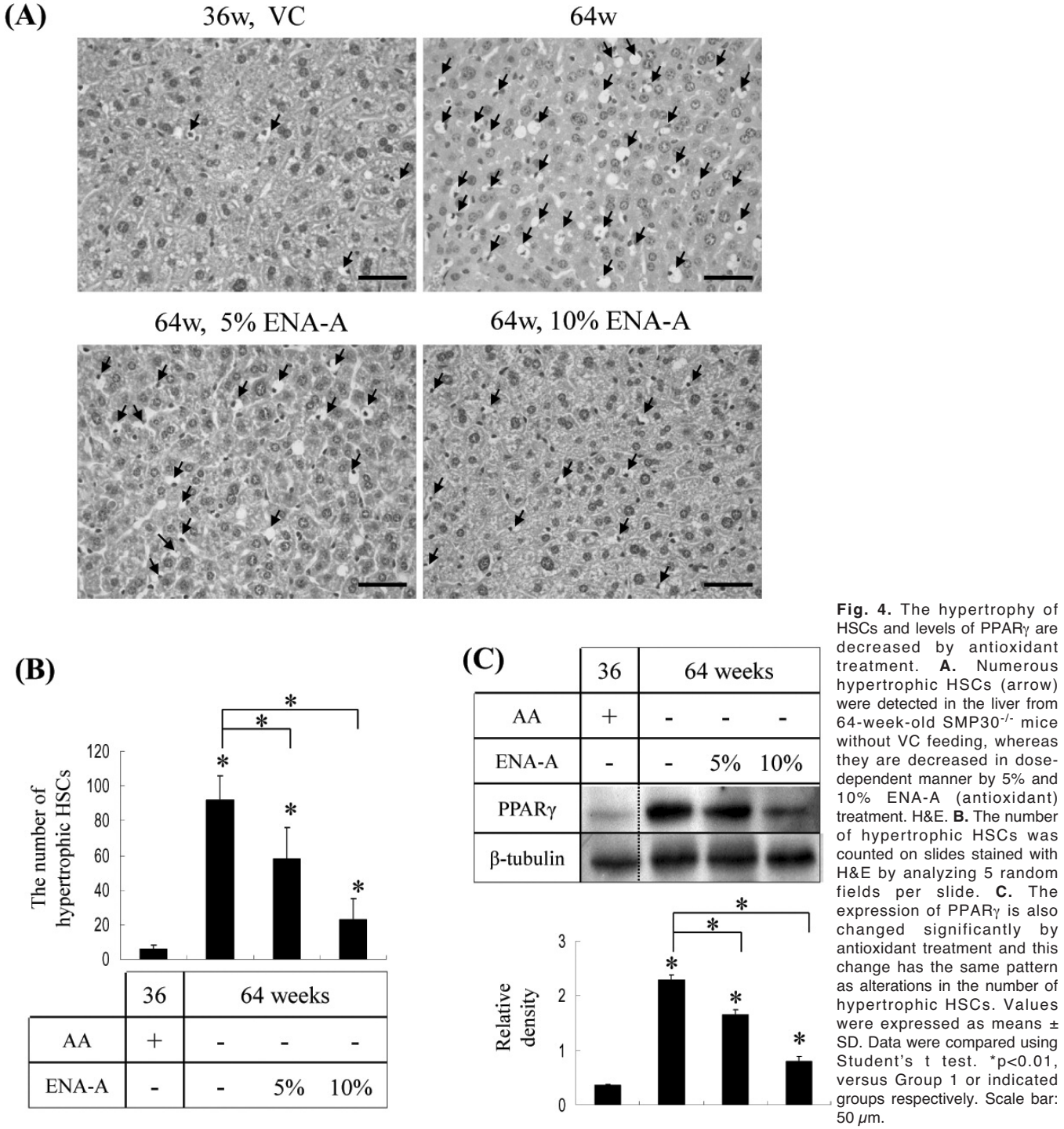

Fig. 4. The hypertrophy of HSCs and levels of PPARγ are decreased by antioxidant treatment. **A.** Numerous hypertrophic HSCs (arrow) were detected in the liver from 64-week-old SMP30$^{-/-}$ mice without VC feeding, whereas they are decreased in dose-dependent manner by 5% and 10% ENA-A (antioxidant) treatment. H&E. **B.** The number of hypertrophic HSCs was counted on slides stained with H&E by analyzing 5 random fields per slide. **C.** The expression of PPARγ is also changed significantly by antioxidant treatment and this change has the same pattern as alterations in the number of hypertrophic HSCs. Values were expressed as means ± SD. Data were compared using Student's t test. *p<0.01, versus Group 1 or indicated groups respectively. Scale bar: 50 μm.

68-week-old mice without VC feeding (Group 2). In agreement with our hypothesis, the number of hypertrophic HSCs was decreased in Group 3 and 4, as compared to Group 2. This decrease is dose-dependent on ENA-A treatment (Fig. 4A,B). The PPARγ expression in the liver had the highest level in the oldest mice not fed with VC (Group 2), whereas it is decreased by VC and by antioxidant treatment in Group 1, 3, and 4 (Fig. 4C). Thus, these studies confirmed that the increase of PPARγ expression with hypertrophic HSCs is an age-related change mediated by oxidant stress in the liver.

Discussion

The liver has long been considered as the tissue that does not have major age-related changes (Popper, 1986). However, recently, senescence of the liver cells has been reported which contribute to functional alterations of the liver (Ito et al., 2007; Krizhanovsky et al., 2008; Le Couteur et al., 2008). In the present study, we used SMP30$^{-/-}$ mice that have redox conditions similar to those initiated in human beings by deficiency of VC synthesis. We have detected an increase of hypertrophic HSCs with lipid droplets in livers of these mice by aging and by VC deficiency. The presence of lipid engorged HSCs was detected in old mice and baboons, and those HSCs were considered to be of an age-related nature (Cogger et al., 2003; Warren et al., 2005). However, there was no specific study that examined this morphological change with HSCs.

The HSC is the primary cell-type in the liver responsible for excess collagen synthesis during hepatic fibrosis (Gressner, 1996). HSCs reside in the Disse's space of normal liver, and have vitamin A (VA) and other retinoids (Friedman, 1993). Following liver injury, the HSCs undergo activation, acquire morphology of myofibroblasts, express α-SMA, and loss in cellular VA stores (Rockey et al, 1992). The increase of VA contents in the liver was observed as one of the age-related changes (Vollmar et al., 2002; van der Loo et al., 2004). VA comprises all retinoids that exhibit the biological features of retinol (Blomhoff et al., 1999). HSCs store 80% of the local retinoids within the whole body as retinyl palmitate in lipid droplets, and regulate both the transport and storage of retinoids under physiological conditions (Senoo, 2004). Retinol circulating in the blood enters the cytoplasm by binding to the receptor located on the cell surface of HSCs. Then it is transported and bound to nuclear retinoic acid receptors, including the retinoic acid receptor (RAR) and the retinoid X receptor (RXR) (Senoo, 2004; Ziouzenkova and Plutzky, 2008). RXR displays its functions as a heterodimer with PPARγ. The PPARγ/RXR heterodimers bind to specific response elements (PPREs) in the promoters of target genes which are involved in the regulation of adipogenesis (Ziouzenkova and Plutzky, 2008). In addition, a previous study showed that VC treatment exhibited a reduction of PPARs (α, γ) expression in mononuclear cells (Kaul and Baba, 2005). Therefore, we speculated that hypertrophic HSCs are associated with an increase of PPARγ expression, and we confirmed this in the present study through microscopic examination and immunoblot analysis.

PPARγ is required for adipogenic transcriptional regulation in HSC quiescence and vitamin A storage (Hazra et al., 2004; She et al., 2005). PPARγ activity is reduced in activated HSCs. The treatment with synthetic PPARγ ligands to activated HSCs suppresses the fibrogenic activity of HSCs (Marra et al., 2000; Miyahara et al., 2000; Galli et al., 2002; Kon et al., 2002). Thus, PPARγ plays a key role in the inhibition of liver fibrosis and in maintenance of HSC quiescence through adipogenic transcriptional regulation. Furthermore, we previously demonstrated the higher expression of PPARγ in isolated senescent HSCs and then showed that VC deficiency ameliorated liver fibrosis by up-regulation of PPARγ expression in SMP30$^{-/-}$ mice (Park et al., 2010). Although there are some studies suggesting that PPARγ might play a pivotal role in cellular senescence (Lu et al., 2005; Gan et al., 2008), the increase of PPARγ expression by aging has not been reported in the liver.

In conclusion, our data demonstrate that VC deficiency accelerates senescence of the liver and that senescent HSCs are hypertrophic and have the accumulation of lipid droplets. The senescent HSCs are in a quiescent state with the increase of PPARγ expression. Furthermore, these alterations are confirmed as age-related changes due to delay by VC and antioxidants. Therefore, we are the first to report the spontaneous increase of PPARγ expression by aging in the liver, so the histological lesion of HSC hypertrophy can be considered as a new marker associated with the evaluation of redox or the senescent condition of the liver.

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