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Ascorbate promotes carbon tetrachloride-induced hepatic injury in senescence marker protein 30-deficient mice by enhancing inflammation☆

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Abstract

The genetic deletion of the senescence marker protein 30 (SMP30) gene results in ascorbate deficiency and the premature aging processes in mice. Apparent liver injury of SMP30$^{-/-}$ mice was less severe than those of wild type (WT) mice, upon chronic CCl_4 injection. The purpose of this study was to investigate the pathophysiology underlying the mild CCl_4 toxicity in SMP30$^{-/-}$ mice. Along with the lower level of serum alanine aminotransferase, the livers of SMP30$^{-/-}$ mice revealed a lesser glycogen depletion, a decrease in c-*Jun* N-terminal kinase (JNK)-mediated inflammatory signaling in parallel with tumor necrosis factor-alpha and interleukin-1 beta, inducible nitric oxide synthase and glutathione peroxidase, and the lower lipid peroxidation as compared to those of WT mice. CCl_4-induced proliferation, measured by the expression of proliferating cell nuclear antigen, was low in SMP30$^{-/-}$ mice as compared with that of WT mice whereas the levels of p21 and Bax were comparable to those of the CCl_4-treated WT mice. Moreover, CCl_4 toxicity in ascorbate-fed SMP30$^{-/-}$ mice was comparable to that of the CCl_4-alone treated WT mice, accompanied by an increase in the above mentioned factors. Conversely, ascorbate partly compensated for the CCl_4-induced oxidative stress in WT mice, indicating that sufficient ascorbate may be required for an antioxidant function under severe levels of oxidative stress. Our data suggest that the restoration of ascorbate-deficiency reverses a sluggish immune system into an activated condition by an increase in JNK-mediated inflammation and free radical cascade; thus leading to accelerated hepatic damage in SMP30$^{-/-}$ mice.

Keywords: Ascorbate-deficient animal; Inflammation; Liver injury; Pro-oxidant; Senescence; SMP30

1. Introduction

Oxygen-derived free radicals are the inevitable by-products of various normal biological reactions and have been implicated in a number of human diseases. Antioxidant compounds have been reported to attenuate hepatic fibrosis by scavenging reactive oxygen species (ROS) arising from hepatitis [1]. Ascorbate is a well-known antioxidant that donates electrons and hydrogen ions to oxygen species or free radicals [2]. However, the liver protective effect of ascorbate has not yet been definitively identified.

SMP30 was discovered as a novel protein in 1992, whose expression decreased with age and is mainly expressed in hepatocytes and the proximal tubular cells of kidneys [3]. Because the expression is reduced by CCl_4 intoxication, it is postulated that SMP30 may play a role in modulating the oxidative stress increased with aging [4]. Furthermore, genetic deletion of SMP30 in murine results in the phenotypic changes mimicking the premature aging processes and shortening of the lifespan as compared with WT mice [4]. Recently, Kondo et al. [5] reported that SMP30 acts as a gluconolactonase in L-ascorbic acid biosynthesis. Mice, like most non-primates species, are able to synthesize a sufficient amount of ascorbate to cover their dietary requirements from glucose through the glucuronate pathway. Glucuronate is converted to L-gulonate, that is, in turn, converted to L-gulonolactone by a lactonase [6]. For this reason, SMP30-deficient mice result in ascorbate deficiency, unless ascorbate is provided. In this study, all experimental animals were supplied with regular rodent chow independently of genetic disparity. Regular rodent chow, containing about 110 mg/kg of ascorbate, is insufficient to maintain normal body functions but enough to inhibit scurvy symptoms in SMP30 deficient mice [5,7,8].

At first, the authors hypothesized that the SMP30$^{-/-}$ mice would be more susceptible to an oxidant, causing a more severe liver injury following CCl_4 injection than WT mice. The apparent liver damage of SMP30 deficient mice, however, was less severe than that of WT mice. Recently, our study demonstrated that the up-regulation of peroxisome proliferators-activated receptor-gamma (PPAR-γ) caused by a lack of ascorbate was the pivotal factor in the mechanisms for attenuated liver fibrosis of SMP30$^{-/-}$ mice [9]. PPAR-γ is an

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important negative regulator not only of hepatic stellate cells activation [10], but also of inflammation [11]. To address the implication of ascorbate-deficiency with attenuated inflammation, the hepatic cellular responses to oxidant stress were compared between WT and SMP30$^{-/-}$ mice in the presence or absence of ascorbate supplementation. In addition, since tumor suppressor p53 is considered as a safeguard for the organism against the detrimental effects of various stimuli [12,13] and also plays a role in cellular senescence [14,15], its implication in the attenuation of CCl_4 toxicity of SMP30$^{-/-}$ mice was examined.

2. Material and methods

2.1. Animals

SMP30 deficient mice backcrossed to the C57BL/6 genetic background for seven generations followed by intercrossing for eight generations [8]. Homozygous female (SMP30$^{-/-}$) and male SMP30 knock-out (SMP30$^{-/y}$) mice were obtained from the Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan, and bred to maintain the SMP30 deficient state under a specific pathogen-free status. For breeding and maintenance, SMP30$^{-/-}$ mice were weaned at 2 weeks of age and fed an L-ascorbic acid (AA)-free diet (PICO 5053 LabDiet, Richmond, IN, USA) and supplied with purified tap water supplemented with 1.5 g/L of ascorbate and 10 μM ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich, St. Louis, MO, USA) ad libitum. The C57BL/6 female WT mice were purchased from Orient Bio (Orient Bio, Seongnam, Korea) and maintained for 1 week before experimental use.

2.2. Experimental design and chronic liver injury

Eight-week-old female C57BL/6 WT mice (n = 24, n = 8 per group) and female C57BL/6 SMP30$^{-/-}$ mice (8–12 weeks of age; n = 15, n = 5 per group) were housed in a stainless-steel wire-mesh cage inside temperature (20°C) and humidity-controlled rooms; they were kept on a 12-h light–dark cycle and provided unrestricted amounts of standard mouse chow with trace amounts of ascorbic acid (5L79 Diet, LabDiet) and freely available purified tap water. Two experimental models were subdivided into three groups; Group 1 was control animals that received the vehicle only [1 ml of olive oil per kilogram of body weight (BW)]; Group 2 (CCl_4 group) received 1 ml olive oil with 10% CCl_4/kg BW; and Group 3 [CCl_{4+} ascorbate (AA) group] received 1 ml olive oil with 10% CCl_4/kg BW and a daily intake of 1.5 g/L ascorbate with 10 μM EDTA. Hepatic fibrosis was induced by intraperitoneal injection of CCl_4 three times weekly for up to 15 weeks and ascorbate was supplemented by drinking water for 16 weeks during the experimental period.

2.3. Samples collection

Animal procedures were conducted in accordance with National Institutes of Health guidelines. Sixteen weeks after the start of experiment, animals were fasted overnight. Blood was collected under diethyl ether anesthesia, and then the animals were euthanized. The liver samples were obtained from multiple lobes followed by either being fixed in a 10 % neutral-buffered formalin solution or by being minced and quick-frozen by immersion in liquid nitrogen.

2.4. Histopathology and immunohistochemistry

The formalin-fixed, paraffin-embedded sections of liver samples were cut into 4-μm thickness and stained using the periodic acid-Schiff (PAS) technique for glycogen identification. Deparaffinized liver sections were immunostained for the inducible nitric oxide synthase (iNOS) with relevant antibody (Stressgen Bioreagents, Victoria, BC, Canada). The antigen–antibody complex was visualized by an avidin-biotin–peroxidase complex solution using an ABC kit (Vector Laboratories, Burlingame, CA, USA) with 3,3-diaminobenzidine (Zymed Laboratories, San Francisco, CA, USA). The sections were counterstained with Mayer's hematoxylin.

2.5. Biochemical measurements

Serum alanine transaminase (ALT) concentrations were analyzed with the IFCC UV method (ADVIA, Bayer, Tarrytown, NY, USA) using a commercial reagent (ALT, Bayer). Serum tumor necrosis factor-alpha (TNF-α) was measured using a mouse TNF-α assay kit (R and D systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Frozen liver fragments were homogenized in a RIPA buffer containing 0.1 mM sodium orthovanadate and protease inhibitor (40 mg liver per 1 ml lysis buffer) by grinding the tissue to a fine powder with liquid nitrogen in a pre-chilled mortar and pestle followed by centrifugation at 10 000×*g* for 20 min. The resulting supernatants without the lipid layer were added to each assay mixture. The ascorbate levels of liver homogenates were measured using an ascorbate quantification kit (BioVision, Mountain View, CA) according to the manufacturer's instructions. Lipid peroxidation (LPO) was assessed by determining the content of hepatic thiobarbituric acid-reactive substances (TBARS) at 550 nm and using 2-thiobarbituric acid (2,6-dihydroxypyrimidine-2-thiol) expressed as nanomoles of the malondialdehyde per milligram of liver.

2.6. Immunoblot analysis

Immunoblot analysis with 40 μg of liver homogenate was performed using a corresponding antibody; anti-iNOS, anti-Cu/Zn superoxide dismutase (SOD1), anti-glutathione peroxidase (GPx) (Assay Designs, Ann Arbor, MI, USA; 1:500), polyclonal anti-catalase (CAT), anti- c-*Jun* N-terminal kinase (JNK) 1, anti-p53, anti-p21, anti-Bax and anti-proliferating cell nuclear antigen (PCNA) (Santa Cruz; 1:200), and a monoclonal antibody for the phosphorylated form of JNK1/2 , stress-activated protein kinase/extracellular signal-regulated kinase kinase (SEK) or p53 (ser-15) (Cell signaling Technology, Danvers, MA, USA; 1:200), polyclonal anti-SMP30 (obtained from Achito Ishigami; 1:5000) and anti-β-tubulin (Sigma; 1:1000).

2.7. RNA extraction and reverse transcriptase-polymerase chain reaction

Total RNAs were extracted from the frozen liver tissues using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, the frozen liver fragments were homogenized in 1 ml of Trizol by grinding the tissue into a fine powder with liquid nitrogen in a pre-chilled mortar and pestle followed by centrifugation at 10,000×*g* for 20 min. Total RNA in the resulting supernatants were separated from protein and DNA by extraction with chloroform and then precipitation with isopropanol. The RNA pellet following washing in 75% alcohol was dissolved in diethylene-pyrocarbonate-treated water. The concentration of RNA was quantified with a Quanti-iT RNA assay kit using the Qubit fluorometer (Invitrogen); 100 ng of total liver RNA was used to prepare cDNA using a random octamer and RT and GO mastermix (MP Biomedicals, Solon, OH, USA) or 1-step AccuPower reverse transcriptase-polymerase chain reaction (RT-PCR) premix (Bionner, Daejeon, Korea) according to the manufacturer's instructions. The forward and reverse primers used were as follows: for TNF-α, 5′-GCTCCCTCTCATCAGTTCCA-3′ and 5′-CAGAGAGGAGGTTGACTTTC-3′ (expected product, 320 bp); for c-*Jun*, 5′-ATGGGCACATCACCACTACA-3′ and 5′-TTTTGCGCTTTCAAGGTTTT-3′ (expected product, 628 bp); for c-*fos*, 5′-GGCTCTCCTGTCAACACACACA-3′ and 5′-GAGGCCACAGAGACATCTCCTC-3′ expected product, 539 bp); for interleukin-1 beta (IL-1β), 5′-GCCCATCCTCTGTGACTCAT-3′ and 5′-AGGCCACAGGTATTTTGTCG-3′ (expected product, 230 bp) and for GAPDH, 5′-ACTCACGGCAAATTCAACGG-3′ and 5′-ACCAGTGGATGCAGGGATGA-3′ (expected product, 483 bp). The amplification product was recovered by 1.5% agarose gel electrophoresis.

2.8. Statistics

The levels of serum ALT and TNF-α, the hepatic levels of ascorbate and LPO were analyzed by analysis of variance with post hoc comparison of the means. The differences between the CCl_4-intoxicated WT and SMP30$^{-/-}$ mice were compared by the Mann–Whitney *U* test. Levels of mRNA of TNF-α and various protein expressions were compared by the Student t-test. Results are expressed as mean or median±S.E. for data among each the indicated treatment groups. $P<.05$ was considered significant. All statistical analyses were performed using the SPSS 14.0 statistical software program.

3. Results

3.1. Ascorbate increases serum ALT and TNF-α levels in CCl_4-intoxicated SMP30$^{-/-}$ mice

Sixteen weeks after a CCl_4 injection every other day, WT mice showed signs of hepatic fibrosis, whereas SMP30$^{-/-}$ mice had a significant attenuation in fibrosis so much as about 60% of that of WT mice, as demonstrated in previous data [9]. The serum ALT levels reflects the severity of liver damage and is widely viewed as a specific indicator of liver necrosis [16]. Liver necrosis was also reduced by about 40% as compared with WT mice, on the basis of serum ALT levels ($P=.034$) (Fig. 1A). Although there was no significant difference in ALT levels between the CCl_4-treated WT mice with and without ascorbate, ascorbate increased ALT levels equal to that of CCl_4-treated WT mice in CCl_4, and ascorbate-fed SMP30$^{-/-}$ mice (Fig. 1A). Proinflammatory cytokine TNF-α was also elevated in sera of ascorbate combined SMP30$^{-/-}$ mice, as compared to those of the CCl_4 alone-treated mice, whereas there was no significant difference in TNF-α levels between the two groups of WT mice (Fig. 1B).

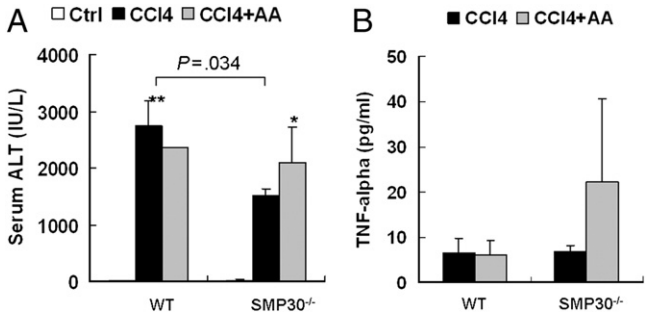

Fig. 1. Serum ALT and TNF-α levels. (A) Hepatic injury as measured by serum ALT levels. CCl_4-intoxicated $SMP30^{-/-}$ mice had significantly lower ALT level compared to that of CCl_4-treatment group of WT mice (P=.034). (B) Serum TNF-α level was determined to evaluate inflammatory response. Statistically significant as compared to the corresponding control group. *P<.05; **P<.01.

3.2. Association of hepatic ascorbate level with liver injury

As shown in Fig. 2A, the mean level of ascorbate in the liver homogenate of CCl_4-intoxicated mice was significantly decreased by approximately 50 % or more when compared to that of the CCl_4-untreated control mice. Unexpectedly, CCl_4 plus ascorbate-treated WT mice had lower hepatic ascorbate levels than the CCl_4 alone-intoxicated WT animals (P=.078) (Fig. 2A), suggesting that exogenous supplementation of ascorbate may suppress the endogenous production of ascorbate. The level of ascorbate coincided with the expression levels of SMP30 in the WT mice (Fig. 2B), indicating that this protein has a direct part in ascorbate synthesis. Interestingly, the hepatic ascorbate levels in $SMP30^{-/-}$ mice was determined to be more or less 70 % than that for WT mice (Fig. 2A). The reason why $SMP30^{-/-}$ mice had this amount of hepatic ascorbate may be explained as follows: as mentioned above, all experimental animals were supplied with regular rodent chow, including about 110 mg/kg of ascorbic acid, independently of genetic disparity. Furthermore, the absence of gluconolactonase may lead to decreased degradation of ascorbate [17]. Taken together, $SMP30^{-/-}$ mice may have a mechanism underlying the ascorbate reservoir in the liver such as increasing uptake or recycle of trace amounts of ascorbate and minimizing its efflux.

In CCl_4-treated WT mice, glycogen deposition in the liver sections was severely depleted as judged using a specific glycogen-staining (PAS staining); CCl_4-induced glycogen depletion was limited to animals supplemented with ascorbate (Fig. 2C). Toxins require adenosine-5'-triphosphate (ATP) (and other energy molecules) to detoxify, depleting the body of oxygen, causing hypoxia and initiating anaerobic glycolysis when using glycogen, whereby glycogen stores are rapidly depleted in the liver [18]. Therefore, hepatic glycogen depletion may be associated with liver injury. In addition, hepatic glycogen stores may be also used in the scavenging of free radicals by generating ascorbate; thus, exogenous ascorbate supplementation may reduce the amount of glycogen depletion in the WT mice (Fig. 2C). In $SMP30^{-/-}$ mice, on the contrary, the CCl_4-induced hepatic glycogen depletion was lower than that of WT mice and increased by ascorbate

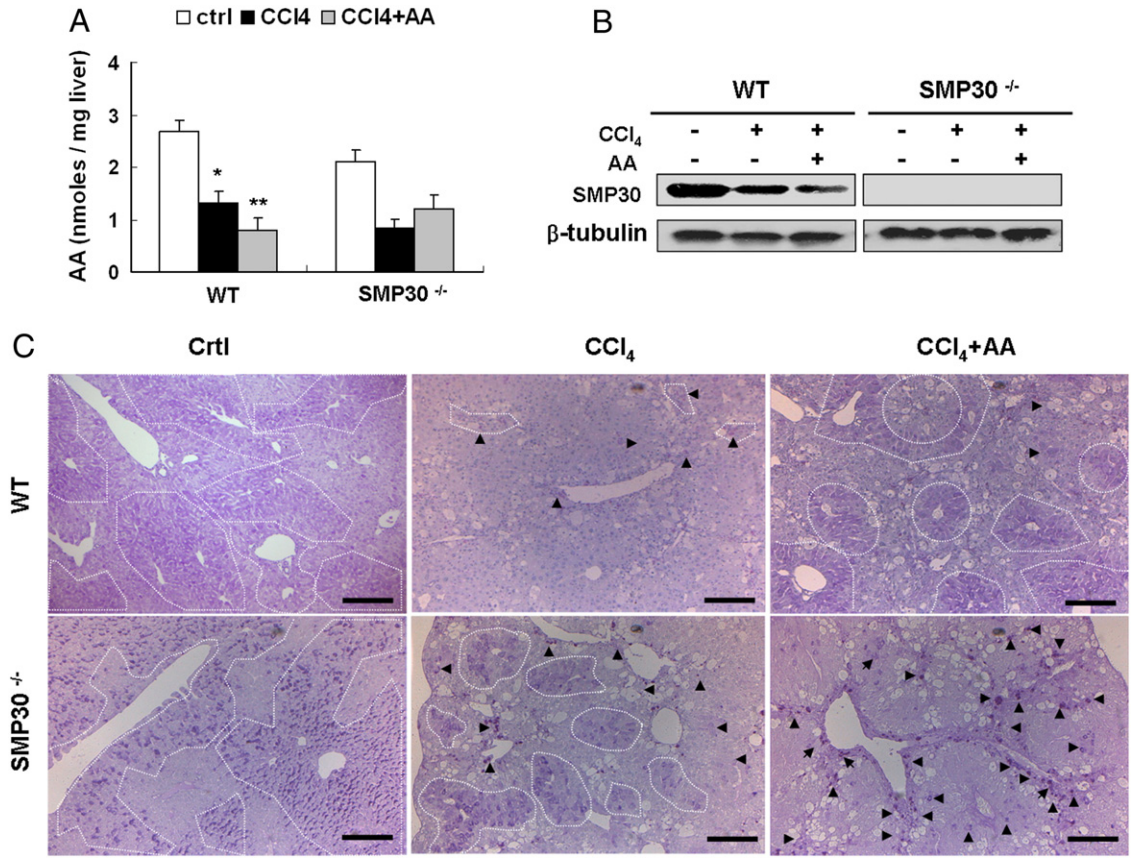

Fig. 2. Effect of CCl_4 intoxication with or without ascorbate supplementation on hepatic ascorbate level and glycogen depletion. (A) Quantification of ascorbate in liver homogenates of experimental animals. Data are the mean±S.E. Statistically significant as compared to corresponding control group. *P<.05; **P<.01. There was a significant difference among the WT groups by Kruskal-Wallis test (P=.006); there was a trend for ascorbate supplementation to reduce hepatic tissue ascorbate level in CCl_4-toxicated WT mice (P=.078). (B) Immunoblot analysis of SMP30 in liver of WT and $SMP30^{-/-}$ mice. The expressions of β-tubulin are shown as loading controls. (C) Liver histology with PAS staining for glycogen deposition. Pas-positive region and cell are represented by white dotted line and arrow head, respectively. Scale bar, 200 μm.

supplementation (Fig. 2C), indicating an increased amount of liver injury. In untreated control SMP30$^{-/-}$ mice, similar amounts of hepatic glycogen were observed when compared to the livers of the control WT mice (Fig. 2C).

3.3. Genetic deletion of SMP30 suppresses JNK1 mediated signal pathway

Fibrosis is a common response to hepatocellular necrosis or injury induced by inflammation [19]. JNK, a stress kinase, has been implicated in oxidative stress-induced liver injury [20]. Thus, CCl_4-induced JNK activation was investigated. CCl_4 induced-JNK1 activation was attenuated by ascorbate supplementation in WT mice (Fig. 3A). In contrast, JNK1 activation was increased by ascorbate administration in the livers of the SMP30$^{-/-}$ mice, indicating that liver injury or liver fibrosis was mediated by JNK signaling. In the immediate upstream kinase, SEK1/MKK4 activation was in accordance with the JNK1 activation (Fig. 3A).

SEK1/MKK4-mediated JNK1 signaling cascade is known to phosphorylate c-Jun that is required for the regulation of inflammatory cytokine gene expression [21]. Expectedly, as shown in Fig. 3B, SMP30 deficient hepatocytes had lower mRNA expression levels of c-*Jun* and c-*fos* in response to CCl_4 as compared to those of the CCl_4-intoxicated WT mice and ascorbate-supplemented SMP30$^{-/-}$ mice. IL-1β, as a downstream target of JNK-mediated inflammatory signaling, was expressed in parallel to pJNK, c-*Jun* and c-*fos* in the CCl_4-alone or in combination with ascorbate.

3.4. Genetic deletion of SMP30 suppresses oxidative stress

The oxidative stress contributes to tissue damage and inflammation which, in turn, causes the increased generation of TNF-α, ROS and nitric oxide (NO) underlying oxidative changes of macromolecules such as DNA, proteins, and lipids. While ascorbate supplementation decreased the CCl_4-induced TNF-α mRNA level in the liver of WT mice, it significantly increased that level in the SMP30$^{-/-}$ mice (P<.05) as shown in sera (Fig. 4A). Concordant with the lower level of TNF-α, the hepatic TBARS level as an indicator of LPO, and iNOS level as a producer of NO, were lower in SMP30$^{-/-}$ mice than in WT mice, and those levels were increased by ascorbate supplementation (Fig. 4B and 4C). Although iNOS originated in macrophages, hepatocytes

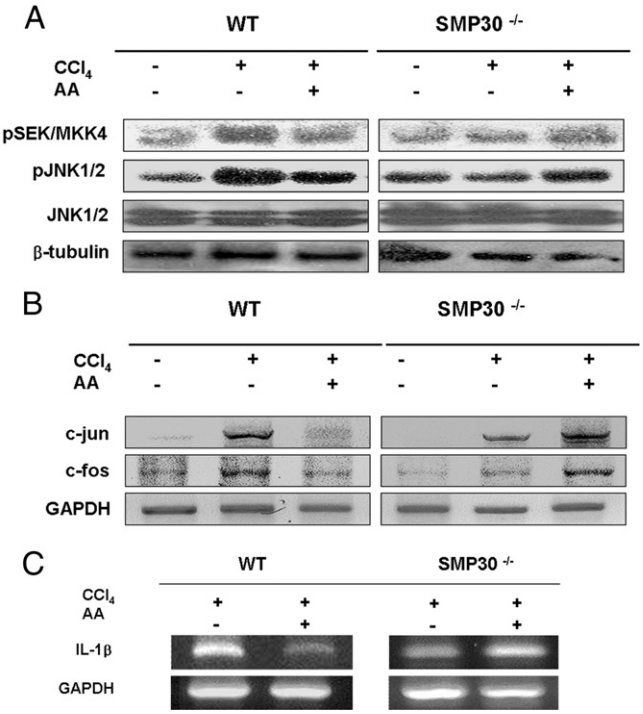

Fig. 3. SEK/JNK signaling and c-*Jun*/c-*fos*, and IL-1β mRNA expression in liver in response to CCl_4 intoxication. (A) Quantitative expression of representative of phospho-SEK, phospho-JNK and JNK were determined by immunoblot analysis. (B) Hepatic level of c-*Jun* and c-*fos* and (C) IL-1β mRNA were measured by RT-PCR. The expressions of GAPDH mRNA are shown as control for relative quantitation of gene expression. Data are representative of at least two experiments.

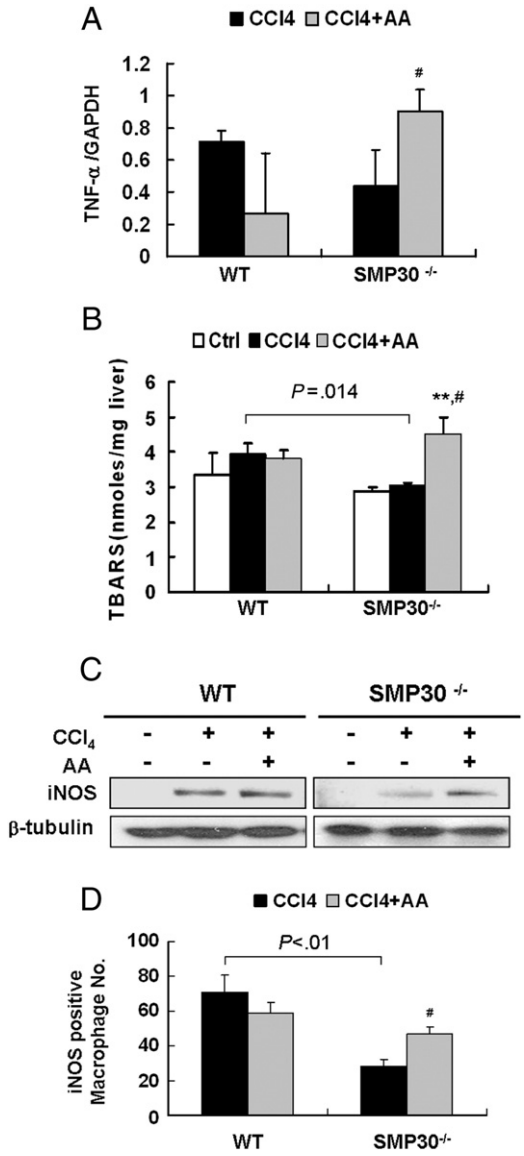

Fig. 4. LPO, TNF-α and iNOS expression. (A) Relative mRNA level of TNF-α was analyzed by RT-PCR. Statistically significant as compared to CCl_4-treatment group of SMP30$^{-/-}$ mice. $^{\#}P$<.05. (B) LPO in liver homogenates of experimental animals was measured by determining the content of hepatic thiobarbituric acid-reactive substances (TBARS) at 550 nm. Statistically significant as compared to the corresponding control group ($^{**}P$<.01); statistically significant as compared to the corresponding CCl_4-treatment group. $^{\#}P$<.05. (C) The expressions of representative of iNOS were determined by immunoblotting in liver homogenates of WT and SMP30$^{-/-}$ mice. The expression of β-tubulin was shown as loading controls. Data are representative of at least 2 experiments. (D) iNOS-positive macrophage infiltration as determined by immunohistochemistry of liver sections and quantification by counting in five fields per slide (original magnification ×400), and three slides were examined for each group. Data are the mean±S.E. Statistically significant as compared to the corresponding CCl_4-treatment group. $^{\#}P$<.05. Statistically significant as compared to CCl_4-treatment group of WT mice (P<.01).

have been shown to express this enzyme [22]. To determine whether the increased iNOS expression by ascorbate is a result of the induction of iNOS in the hepatocytes or in the macrophages, we identified the location and distribution of iNOS in immunostained liver sections. iNOS expression in the liver sections was mainly observed at macrophages around the central vein and along the fibrous septa. The iNOS positive macrophages in SMP30$^{-/-}$ mice were reduced by 60% (P<.01) compared to those in WT mice (Fig. 4D). While ascorbate supplementation appeared to reduce the numbers of macrophages in the liver sections of WT mice by 20% (P>.05), it increased those numbers in the SMP30$^{-/-}$ mice by 30 % (P<.05) as compared with those of the relevant CCl_4 alone treatment group (Fig. 4D).

CCl_4-intoxication significantly depleted SOD1 expression in the livers of WT mice, whereas hepatic levels of CAT and GPx, remained unchanged (Fig. 5A and B). Ascorbate supplementation limited SOD1-depletion. In SMP30$^{-/-}$ mice, antioxidant proteins including SOD1, CAT and GPx remained nearly unchanged upon CCl_4-induced oxidative stress, but ascorbate increased those levels. Most notably, the GPx level was significantly increased by ascorbate supplementation (P<.05). Taken together, it is likely that the antioxidant enzymes are increased in conformity to an increase in ROS and NO production that is, at least in part, due to the enhanced inflammations by ascorbate supplementation.

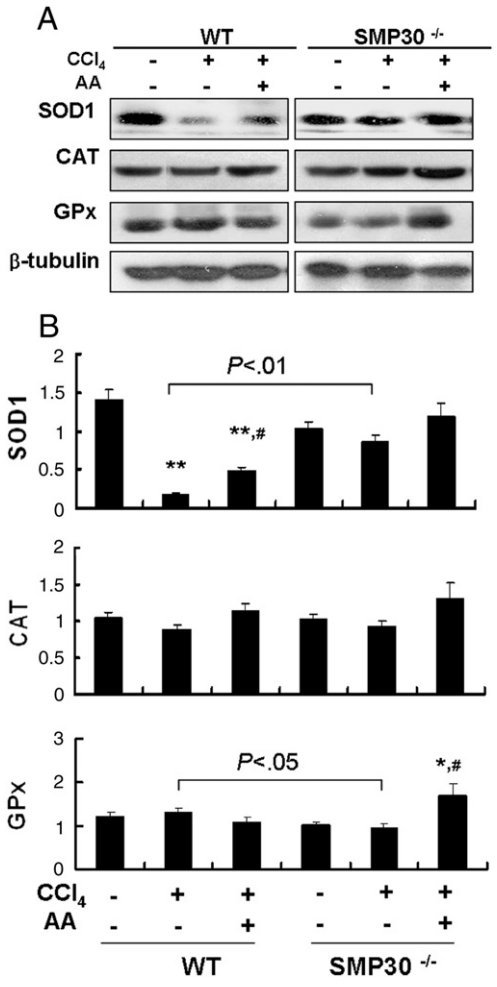

Fig. 5. Differential expression of SOD1, CAT and GPx in response to CCl_4 intoxication. (A) The expressions of representative of SOD1, CAT or GPx was determined by immunoblotting in liver homogenates of WT and SMP30$^{-/-}$ mice. The expression of β-tubulin was shown as loading controls. (B) Relative ratios of SOD1, CAT or GPx to β-tubulin were measured with Image J software. Data are representative of at least two experiments. Values are the mean±S.E. Statistically significant as compared to corresponding control group. $^{*}P$<.05; $^{**}P$<.01. Statistically significant as compared to corresponding CCl_4-treatment group. $^{\#}P$<.05.

3.5. Genetic deletion of SMP30 up-regulates p53-mediated cell arrest

Tumor suppressor protein p53 has been implicated in anti-inflammatory responses including the down-regulation of iNOS and cyclooxygenase-2 pathways [23]; moreover, recent work of Krizhanovsky et al. [24] revealed that a senescence program mediated by p53 limited the fibrogenic response to acute tissue damage. Furthermore, an increase in SOD1 and a decrease in GPx may give rise to the accumulation of H_2O_2 that has been the most commonly used inducer for stress-induced premature senescence [25]. Thus, the effects of ascorbate deficiency on p53-mediated signaling were investigated.

The p53 expression was increased in response to oxidative stress in both WT and SMP30$^{-/-}$ mice (Fig. 6A). In the case of SMP30$^{-/-}$ mice, hepatic p53 expression was increased in the control mice group, as well as CCl_4-intoxicated group, indicating that an ascorbate defect may impose an oxidative burden upon the SMP30$^{-/-}$ mice. The p21 expression is normally up-regulated by p53, and its elevation can lead to premature senescence [26]. The p21 expression was significantly increased in SMP30$^{-/-}$ mice in accordance with the phosphorylation on serine 15 of p53 (Fig. 6A). Maintaining cells in the G1 phase by p53 mediated p21 protects cells from replicating damaged DNA and facilitates DNA repair [27]. If p21 induced-growth arrest persists, regenerative capacity (or proliferation) will decline, causing cellular senescence and apoptosis. PCNA functions both in DNA replication and in DNA repair and it has been simultaneously induced along with p53 and p21 upon genotoxic stress [28]. Bax is an apoptosis related protein and a transcriptional target for p53 [29] in responses to oxidative stress [30]. Based on these studies, we next investigated the hepatic expressions of PCNA and Bax. The expression of PCNA was lowered in SMP30$^{-/-}$ mice as compared to those of the ascorbate-producing WT mice or ascorbate-supplemented SMP30$^{-/-}$ mice after CCl_4 injection (Fig. 6B). Expectedly, the Bax expression was increased upon CCl_4 intoxication (Fig. 6B). An ascorbate supplementation slightly lowered the level of Bax in WT mice, whereas it increased the Bax expressions in SMP30$^{-/-}$ mice in parallel to the p21 expressions (Fig. 6B).

4. Discussion

Ascorbate supplementation slightly reduced CCl_4-induced liver fibrosis in WT mice; in contrast, it accelerated liver fibrosis in CCl_4-intoxicated SMP30$^{-/-}$ mice that was less severe than in WT mice, as shown in our previous work [9]. Dietary ascorbate consumption decreases oxidative stress and the inflammatory status for healthy humans [31], as shown in the results from the CCl_4-injured WT mice. Banhegyi et al. [32] revealed that ascorbate synthesis caused H_2O_2 production, accompanied by almost equimolar glutathione consumption, in isolated murine hepatocytes. Low hepatic ascorbate level and maintaining the glycogen deposition in WT mice supplemented with ascorbate may be associated with utilization of exogenous ascorbate rather than endogenous one, causing reducing oxidative stress (Fig. 2). These results indicate that SMP30 may have a potential role in the modulation of the immune response in tissue injury and repair by up-regulating or down-regulating the ascorbate synthesis depending on levels of oxidative stress.

Hepatic fibrosis results in the excessive deposition of collagen type I, and ascorbate, as a cofactor of prolyl hydroxylase, is integral in this process [33]. Therefore, ascorbate deficiency may, in part, delay fibrogenesis in SMP30$^{-/-}$ mice by destabilization of collagen fiber. However, judged by several markers related liver injury, SMP30$^{-/-}$ livers had resistance to CCl_4 intoxication as compared to that of WT

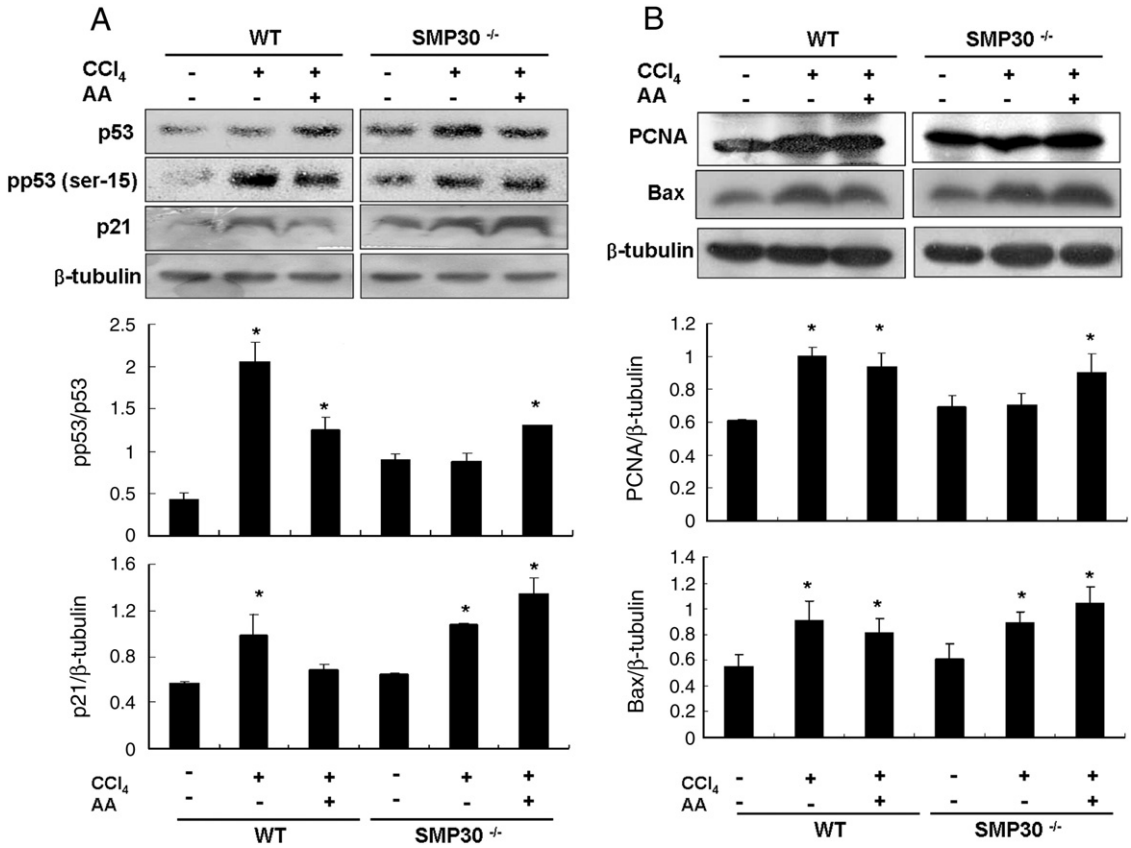

Fig. 6. An increase in p53-mediated signaling pathway in the liver of SMP30$^{-/-}$ mice. (A) Hepatic expressions of p53, phopho-p53 (Ser-15), p21 and β-tubulin were determined by immunoblot analysis. The expressions of β-tubulin are shown as loading control. (B) Expression levels of PCNA and Bax. The expressions of β-tubulin are shown as loading control. Relative ratios of phosphor-p53 to p53 and p21, Bax or PCNA to β-tubulin were measured with Image J software. Data are representative of at least two experiments. Values are the mean±S.E. Statistically significant as compared to corresponding control group. $^{*}P<.05$. Statistically significant as compared to corresponding CCl_4-treatment group ($P<.05$).

mice. According to our unpublished data, other antioxidant supplements like an angiotensin-type II receptor blocker also accelerated CCl_4-induced liver fibrosis in SMP30$^{-/-}$ mice, whereas they lowered CCl_4-induced liver fibrosis in WT mice [34]. Thus, the role of ascorbate, other than in collagen production, needs to be elucidated.

Superoxide anion and nitric oxide arise in cell metabolism to yield peroxynitrite that can directly damage plasma and intracellular membranes resulting in increased LPO [35]. Lei et al. [36] has demonstrated that GPx protects against superoxide-induced cell death; however, it promotes peroxynitrite-induced cell death. An increase in both GPx and iNOS expression by ascorbate supplementation may accelerate the level of peroxynitrite toxicity in SMP30$^{-/-}$ mice, judged by an increased LPO. Ascorbate has pro-oxidant effects in the presence of iron in vitro, by maintaining iron in a reduced state (Fe^{2+}), leading to the production of hydroxyl radicals and lipid alkoxyl radicals, namely Fenton's reaction [37]. According to the work of Premkumar et al. [38], ascorbate (15 g/kg diet) increased LPO by about 60%, coupled with an increase in the uptake of iron by 12 % in the livers of C3H/He wild-type mice fed the low-iron diet (100 mg/kg diet), as compared with those of mice without ascorbate supplementation. In contrast, the high-iron diet (300 mg/kg diet) significantly increased LPO independently of ascorbate supplementation, indicating that ascorbate increases the oxidative stress but does not increase it depending on the iron concentration in the liver. Although we did not measure hepatic iron concentration, ascorbate supplementation may increase LPO by increasing iron uptake and maintaining iron in a reduced state followed by Fenton's reaction in the SMP30$^{-/-}$ mice. According to a recent study [39], ascorbate sustains neutrophil NOS expression, catalysis, and oxidative burst, thereby reinforcing oxidative microbicidal actions of the neutrophils. An animal study by Victor et al. [40] has shown that macrophages and lymphocytes need an appropriate level of antioxidants, such as ascorbic acid, under oxidative stress conditions. More interestingly, the studies by Lee et al. [41] revealed that *Helicobacter pylori* infections resulted in comparable gastritis and premalignant lesions in WT mice and Gulo$^{-/-}$ mice (ascorbate deficient mice) fed high dose of ascorbate, but the lesions were less severe in Gulo$^{-/-}$ mice fed low-doses of ascorbate; in contrast, less severe lesions of *H.pylori* infected Gulo$^{-/-}$ mice correlated with reduced Th1-associated IgG2 and higher *H.pylori* colonization levels. We also confirmed mild liver injury with higher *H.pylori* colonization levels in CCl_4 plus *H. pylori*-treated SMP30$^{-/-}$ mice whereas ascorbate supplementation caused more severe liver injury with lower colonization levels in CCl_4 plus *H. pylori*-treated SMP30$^{-/-}$ mice [our unpublished data]. Thus, ascorbate appears to be necessary for the inflammatory responses against microbial or viral infections; however, increased oxygen species during inflammation may augment the lesions at the site of infection, especially under the ascorbate deficient conditions such as aging, stress, smoking, and several other pathological states.

Concordantly, TNF-α, c-*Jun*, c-*fos*, IL-1β, and TNF-α mRNA levels were significantly increased in ascorbate supplemented SMP30$^{-/-}$ mice, coupled with an increase in JNK activation. JNK plays a critical role in acetaminophen-induced liver injury, which primarily involves hepatocyte necrosis and JNK inhibition dramatically ameliorated the liver injury [20]. In CCl_4-induced liver injury, JNK has been known to stimulate the production of proinflammatory cytokines via activator protein 1 [42] and/or nuclear factor κB [43] and prolonged JNK activation promotes cell death (necrosis and/or apoptosis) depending

on cell type and stimulus [44,45]. Apoptosis is critical for the clearance of damaged and mutated cells in a way that does not disturb the surrounding tissue [46]. Necrosis, coupled with inflammation is also necessary for the removal of viral disease, injury-induced inflammation and cancer [47]. The data gleaned from this study showed ascorbate supplementation increased p21 and Bax expressions, indicating that it may augment cell arrest and apoptosis in the CCl_4-intoxicated livers of SMP30$^{-/-}$ mice. Vissers et al. [48] demonstrated the role of ascorbate as a modulator of apoptosis; oxidative inhibition of apoptosis induced by chronic oxidative insults may be abrogated by ascorbate, allowing apoptosis to proceed.

Several lines of evidence suggest that p53 can stimulate the expression of genes that reduce oxidative stress [13,49]. On the contrary, some suggest that p53 transactivates ROS-generating enzymes including quinine oxidoreductase, proline oxidase, Bax, p53 up-regulated modulator of apoptosis and p66^{shc} that lead to oxidative stress and consequently to apoptosis [50–52]. In unstressed circumstances, p53 is degraded via murine double minute 2-mediated ubiquitination [53], which is blocked by post-translational modification of p53 and in turn, causes the accumulation of p53 [54]. CCl_4 intoxication caused p53 expression and its phosphorylation at Ser-15. Ascorbate supplementation further increased or decreased the p53 phosphorylation at Ser-15, thereby allowing or limiting p53 mediated cellular processes including transactivation of anti-oxidant genes or pro-oxidant genes depending on the cellular redox status, which may explain, in part, the reason why ascorbate plays a role as an anti-oxidant or pro-oxidant in the CCl_4-treated WT or SMP30$^{-/-}$ mice.

Furthermore, there was a tendency for PCNA expression to be suppressed in SMP30$^{-/-}$ mice as compared to that of WT mice in response to CCl_4, whereas there was a tendency for Bax expression to be similar or rather increased in SMP30$^{-/-}$ mice. In the view of the maintenance of cell number homeostasis in normal tissues by regulating the balance between proliferation and apoptosis in response to a toxic insult, suppressed proliferation and a similar extent of apoptosis in hepatocytes in SMP30$^{-/-}$ mice may give rise to a subsequent risk of the loss of regenerative capacity that progresses to cellular senescence [55]. In accordance with this notion, ascorbate is reported to enhance the generation of induced pluripotent stem cells, at least in part, by alleviating cell senescence [56], indicating ascorbate may play a critical role in the production of progenitor cells for regeneration. Therefore, the injured hepatocytes may be predisposed to undergo senescence or degenerative transformation instead of repair in subjects with chronic ascorbate-deficiency, although the inflammation and fibrosis are less severe as compared to ascorbate-sufficient ones.

In summary, first, ascorbate may act as a pro-oxidant in ascorbate-deficient mice by activating ROS mediated SEK/JNK signaling pathway and sluggish immune responses, causing an increase in c-*Jun* / c-*fos*, IL-1β, TNF-α and iNOS, thereby leading to an increase in ROS and NO production along with a more serious hepatic injury. Second, ascorbate-deficiency may pose an oxidative burden on SMP30$^{-/-}$ mice, which leads to an increase in p53 and p21 expressions followed by repressed cell proliferation. Therefore, increased cell arrest under ascorbate-deficient conditions against chronic oxidative stress may result in the accumulation of damaged cells, causing cellular senescence and/or neoplastic transformation, which might be abrogated by ascorbate supplementation through facilitating the inflammation and apoptosis.

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