Cu,Zn-SOD deficiency induces the accumulation of hepatic collagen

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**ABSTRACT**

Nonalcoholic fatty liver disease (NAFLD) is one of the most prevalent chronic diseases, and results in the development of fibrosis. Oxidative stress is thought to be one of the underlying causes of NAFLD. Copper/zinc superoxide dismutase (SOD1) is a primary antioxidative enzyme that scavenges superoxide anion radicals. Although SOD1 knockout (KO) mice have been reported to develop fatty livers, it is not known whether this lack of SOD1 leads to the development of fibrosis. Since the accumulation of collagen typically precedes liver fibrosis, we assessed the balance between the synthesis and degradation of collagen in liver tissue from SOD1 KO mice. We found a higher accumulation of collagen in the livers of SOD1 KO mice compared to wild type mice. The level of expression of HSP47, a chaperone of collagen, and a tissue inhibitor (TIMP1) of matrix metalloproteinases (a collagen degrading enzyme) was also increased in SOD1 KO mice livers. These results indicate that collagen synthesis is increased but that its degradation is inhibited in SOD1 KO mice livers. Moreover, SOD1 KO mice liver sections were extensively modified by advanced glycation end products (AGEs), which suggest that collagen in SOD1 KO mice liver might be also modified with AGEs and then would be more resistant to the action of collagen degrading enzymes. These findings clearly show that oxidative stress plays an important role in the progression of liver fibrosis.

**Introduction**

Nonalcoholic fatty liver disease (NAFLD) is closely associated with obesity, insulin resistance, the metabolic syndrome and diabetes. Due to the increasing incidence of obesity, NAFLD has become a worldwide health concern. NAFLD ranges from steatosis to nonalcoholic steatohepatitis (NASH), which can progress to cirrhosis of the liver. Hepatic fibrosis is the result of an excessive production of collagen caused by the deregulation of physiological wound healing and/or a decreased level of collagen degradation. Activated hepatic stellate cells (HSCs)/myofibroblasts produce and secrete \( \alpha \)-smooth muscle actin (\( \alpha \)-SMA) and collagen as a major source of extracellular matrix, after which, liver fibrosis develops [1–4]. Matrix metalloproteinases (MMPs) play a major role in the degradation of collagen, but the activities of the MMPs are regulated by tissue inhibitors of metalloproteinase (TIMP). If TIMPs are predominant over MMPs, then the degradation of collagen would be expected to be reduced. Therefore, an assessment of MMP and TIMP levels is important in terms of our understanding of fibrogenesis. Moreover, when collagen is glycated and modified with advanced glycation end products (AGEs), it is easily cross-linked and stiffened [5,6]. \( \text{N}^\text{e}-(\text{Carboxymethyl})\text{arginine (CMA) and N}^\text{e}-(\text{Carboxymethyl})\text{lysine (CML are typical AGE components and CMA is found in glycated collagen in diabetes [7]. AGEs are formed by the reactions not only between proteins and sugars, but also between proteins and carbonyl compounds that are produced by lipid peroxidation [8,9].}

A two-hit model has been proposed to explain the progression of simple fatty liver disease to NASH. The first hit is the accumulation of excessive fat in the liver, and second is the development of oxidative stress [10,11]. In fact, in many clinical studies, elevated systemic markers of oxidative stress and lipid peroxidation have been found in patients with NAFLD [12–15]. Several therapeutic strategies targeting oxidative stress decreased the progression of NAFLD. Oliveira et al. showed a marked effect of vitamin C in the prevention of NAFLD in Wister rats [16]. \( \text{N-Acetylcysteine, which maintains GSH levels, improved ischemia-reperfusion...
injury of rat steatotic livers [17,18]. Therefore, oxidative stress in the liver appears to play a central role in the progression of steatosis [19].

Superoxide dismutase (SOD) plays a protective role in cells by catalyzing the converting of the superoxide anion radical into molecular oxygen and hydrogen peroxide. SOD actually consists of three isozymes: copper/zinc SOD (SOD1), which is localized in the cytosol, the nucleus, and the mitochondrial inter membrane space, manganese SOD (SOD2), which is localized in the mitochondrial matrix, and extracellular-SOD (SOD3). Mutations in SOD1 are associated with the development of familial amyotrophic lateral sclerosis (FALS), a type of neurodegeneration that is characterized by the selective death of motor neurons. Although transgenic mice that express the mutant human SOD1 linked with FALS death of motor neurons. Although transgenic mice that express the mutant human SOD1 linked with FALS develop a progressive ALS phenotype [20,21], SOD1 knockout (KO) mice grow normally and exhibit no clear abnormalities except for a small body size and anemia [22]. We recently reported that glutathione S-transferase A4 is induced in SOD1 KO mice kidneys and that this induction is one of the compensatory regulatory systems for protecting cells from oxidative stress in young SOD1 KO mice [23]. However, adult or older SOD1 KO mice exhibit hearing loss [24], and female infertility [25]. In addition, SOD1 KO mice show a lipid accumulation in the liver (this study, [26,27]). As a cause of fatty liver in SOD1 KO mice, an increase in fatty acid synthesis [27] and a decrease in apolipoprotein B-100 levels [26] compared to wild type mice (WT mice) have been reported. A decrease in apolipoprotein B-100 would lead to the impaired lipoprotein secretion from the liver. The development of hepatocellular carcinomas as the result of an SOD1 deficiency has also been reported [28]. In addition, SOD1 KO mice exhibit alterations in iron metabolism in livers [29–31] and kidneys [32]. Increment of hepatic iron concentrations in 3-month-old SOD1 KO mice [29] and mild iron deposits in 1-year-old SOD1 KO mice livers [31] were observed. Elevated hepatic iron leads to oxidative stress via Fenton reaction and may increase the risk of progression of liver diseases such as NASH and hepatic carcinoma [33]. However, the FALS associated mutant SOD1 overexpressed mice also showed fatty liver [34]. These reports prompted us to investigate possible relationships between oxidative stress, SOD1 levels and NAFLD/NASH by using SOD1 KO mice, WT mice, and human SOD1 overexpressed mice (SOD1 Tg mice).

The findings reported in this study indicate that in SOD1 KO mice that were fed a normal diet, the accumulation of collagen in the liver was higher than that in WT mice and SOD1 Tg mice. As a reason for this, we report here that excess collagen accumulation and an MMP/TIMP imbalance leads to the progression to fibrosis in fatty livers in SOD1 KO mice.

Materials and methods

Reagents and antibodies

All chemicals used in this study were purchased from either Wako Pure Chemical Industries Ltd. (Osaka, Japan), Nacalai Tesque, Inc. (Kyoto, Japan), or Sigma-Aldrich (St. Louis, MO), unless otherwise specified, and were of the highest grade available. Polyclonal antibodies against mouse α-SMA and mouse HSP47 were purchased from Gene Tex (San Antonio, TX). Polyclonal antibodies against mouse MMP9 and mouse TIMP1 were purchased from R&D (Minneapolis, MN). Polyclonal antibody against mouse CMA was purchased from Cosmo bio Co., Ltd (Tokyo, Japan). Monoclonal antibody against mouse CML was purchased from TransGenic Inc (Fukuoka, Japan). Polyclonal antibody against mouse β-Actin was purchased from Cell signaling (Danvers, MA).

Experimental animals

The mice used for this study were derived from breeding pairs of heterozygous SOD1-deficient mice obtained from the Jackson laboratory. The mice were backcrossed with C58BL/6j mice more than seven times before the experiments. All breeding and genotyping was performed in a virus- and pathogen-free barrier facility at the Hyogo College of Medicine. The animals were maintained under an artificial 12/12-h light/dark cycle, as well as a constant temperature of 20–22 °C, and had free access to food and distilled water. The genotype of each mouse was verified by the polymerase chain reaction (PCR) of DNA isolated from a tail biopsy, as described in The Jackson Laboratory Web site. All experiments were carried out using 10–12-week-old male mice. All experiments were approved by the Hyogo College of Medicine Animal Care and Use Committee.

Immunohistochemical analysis

Each mouse was deeply anesthetized and transcardially perfused with phosphate buffer saline (PBS), followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Liver tissue was embedded in paraffin, cut into 7-mm-thick sections, and then subjected to immunohistochemical analysis. Sections were deparaffinized and then washed in PBS. Normal serum homologous with the blocking reagent. Tissue sections were incubated with anti-α-SMA, anti-HSP47 and anti-CMA for 18 h at
4 °C. The bound antibodies were visualized by the avidi–biotin–immunoperoxidase complex (ABC) method using an appropriate Vectastain Elite ABC rabbit IgG kit (Vector Laboratories, Burlingame, CA) and 3,3'-diaminobenzidine tetrahydrochloride as the chromogen (DAB). Endogenous peroxidase activity was quenched by incubation with 3% H2O2 for 30 min after the secondary antibody treatment.

**Histological studies**

For histological observations, we stained formalin fixed, paraffin-embedded liver sections (5 μm thickness). Sections were subjected to standard hematoxylin/eosin (HE) staining and Sirius Red F3BA (Chondrex, Redmond, WA) using standard protocols. We stained frozen liver sections (5 μm thickness) with Oil Red O (Nacalai Tesque) for 15 min at room temperature, then washed with water. HE staining was used to observe pathological structures in the livers. Sirius Red staining was used to observe collagen and Oil Red O staining was used to observe the lipid droplets.

**Biochemical analysis**

To extract lipid for the assay of total cholesterol (TC) and triglycerides (TG), 50 mg of liver was mixed with 0.5 ml PBS and homogenized. After the homogenate was extracted with chloroform methanol (5:2.5 ml) and centrifuged, the lower layer was transferred into tube, then dried and dissolved in ethanol. Concentration of TC and TG were measured using Wako test kits (Wako Pure Chemical Industries Ltd.). Blood glucose levels were measured using Lab Gluco (FORA, Tokyo, Japan).

**Collagen assay**

The QuickZyme total collagen assay kit (QuickZyme Biosciences, Leiden, Netherlands) was used for the assay following the manufacturer’s protocol. The total collagen assay is based on the detection of hydroxyproline. In a typical experiment, the measurement of collagen was started by the complete hydrolysis of 200 mg of wet tissue/ml samples in 6 M HCl at 95 °C for 20 h in screw-capped tubes. After the hydrolysis procedure, the tubes were cooled to room temperature and then centrifuged for 10 min at 13,000 g. The supernatants were mixed with assay buffer in 96-well plates and incubated for 60 min at 60 °C. After the incubation, the plate was read at 570 nm.

**ELISA**

The mouse liver homogenate used the following lysis buffer: 200 mM NaCl, 5 mM EDTA, 10 mM Tris, 10% glycine, 1 mM PMSF, 1 μg/ml leupeptin and 28 μg/ml aprotinin (pH 7.4). The samples were centrifuged twice (1500 g at 4 °C for 15 min). The supernatant was assayed for contents of myeloperoxidase (mouse MPO ELISA kit; Hybritech Biotech, Uden, Netherlands).

**Western blotting**

Protein concentrations were determined using a BCA protein assay kit (Pierce, Rockford, IL) with bovine serum albumin as the standard. Protein samples (2 μg) were size-fractionated on a 10% sodium dodecyl sulfate polyacrylamide (SDS) gel and transferred to a Trans-Blot nitrocellulose membrane (Whatman, Dassel, Germany). The membrane was briefly rinsed with Tris-buffed saline containing 0.1% Tween 20 (TBS-T) and then incubated for 1 h at room temperature in TBS-T containing 5% skim milk (Difco, Detroit, MI). The blocked membrane was incubated with an appropriate primary antibody for 12 h at 4 °C. Horseradish peroxidase-conjugated anti-rabbit or -mouse IgG (Zymed Laboratories Inc., South San Francisco, CA) was used as the secondary antibody, and proteins were visualized using an ECL Western blotting detection system (GE Healthcare, Little Chalfont, UK).

**RNA extraction and RT-PCR**

Total RNA was isolated from liver samples using the Sepazol (Nacalai Tesque) reagent. The expression of different mRNAs of interest was evaluated by the reverse transcriptional polymerase chain reaction (RT-PCR) using a High RNA-to-cDNA Kit (Applied Biosystems, Branchburg, NJ).

**Real time PCR**

Relative semi-quantitative analysis of mRNA was performed by PCR using the specific primer sets listed below

| Gene    | Forward primer (5' to 3') | Reverse primer (5' to 3') |
|---------|---------------------------|---------------------------|
| HSP47   | 5'-AAGACCAGGGGCTGGAGAACATCC-3' | 5'-TCTGCGATTTGTCTCTCGGGA-3' |
| MMP2    | 5'-CAACGGTCGGGAATACAGCAG-3' | 5'-CCAGGAAATGTAAGGGGAA-3' |
| MMP9    | 5'-GCTGACTACGATAAGGACGGC-3' | 5'-AGGAAGACGAAGGGGAAACG-3' |
| TIMP1   | 5'-GCATCCTTGGAATCTCTCAGC-3' | 5'-GCCGTTCTGGGACTTGGGC-3' |
| TIMP2   | 5'-GCCAAGACGAGGGTACAGGAA-3' | 5'-GGGGAGGAGATGTAGCAAGGG-3' |
| TIMP3   | 5'-GCCTCATTATTACCACTCAG-3' | 5'-CTGATAGCCAGGGTACCCAAA-3' |
| TIMP4   | 5'-CTTATCTGCCCTGGCCTCACG-3' | 5'-CTTATCTGCCCTGGCCTCACG-3' |
Amplification conditions were 1 min at 95°C for the initial denaturation, followed by 25 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. The PCR products were subjected to electrophoresis on a 3% agarose gel and then stained with ethidium bromide. AGEs receptor (RAGE) mRNA was measured by real time PCR (7900 PCR system, Applied Biosystems), using a RAGE Taqman probe (Life Technologies, Carlsbad, CA). IDs of primers for mouse RAGE and β-actin gene were Mm01134790_g1 and Mm00607939_s1, respectively. All data were normalized by β-actin expression.

**Results**

**Lipid levels are increased in SOD1 KO mice livers**

We first confirmed the normal expression levels of SOD1, no SOD1 expression, and the expression of human SOD1 in WT, SOD1 KO and SOD1 Tg mice livers, respectively (Figure 1A). To examine the state of the livers of three different types of mice, myeloperoxidase (MPO) protein levels, which reflect the level of inflammation and/or oxidative stress that is associated with numerous disease such as atherosclerosis, were measured by ELISA. The higher contents of MPO in SOD1 KO mice livers and the lower MPO levels in SOD1 Tg mice livers compared with WT mice indicate a higher degree of inflammation as the result of the lack of SOD1 and a lower inflammation caused by a higher expression of human SOD1, respectively (Figure 1B). In contrast, there were no significant differences in H2O2 levels between three mice livers (Supplementary Figure S1). To confirm the accumulation of lipid in SOD1 KO mice livers, we stained them with Oil Red O. As shown in Figure 1(C), we observed many lipid droplets in the livers of SOD1 KO mice. Interestingly, the lipid droplets were also detected in SOD1 Tg mice livers, although the amount was much lower in the case of SOD1 KO mice. In contrast, there were only few lipid droplets in the WT mice livers. Moreover, HE staining revealed the presence of numerous hepatic vacuoles in SOD1 KO mice. To check the basic state of the liver, TC and TG levels in the mice livers were measured. The concentration of TC were about 1.9 mg/g in WT mice, 2.5 mg/g in SOD1 KO mice, and 2.1 mg/g in SOD1 Tg mice, respectively (Figure 1D-a), indicating that the concentration of TC was elevated in SOD1 KO mice, but the concentration of triglycerides were not changed among these mice (Figure 1D-b).

**SOD1 KO mice livers produce collagen**

Morphological differences in liver fibrosis in liver sections were visualized by staining with Sirius Red. Although the WT and SOD1 Tg mice exhibited no sign of fibrosis, pericellular fibrosis around the central veins was observed in SOD1 KO mice livers (Figure 2A). To
ascertain fibrosis in SOD1 KO mice livers, the concentrations of collagen were determined by an assay based on the detection of hydroxyproline residues in collagen. The concentrations of collagen of SOD1 KO mice livers were approximately twofold higher than of those of WT mice livers (Figure 2B). We next measured the expression levels of HSP47 and α-SMA in these mice livers. HSP47 and α-SMA are reliable, widely used fibrosis markers because HSP47 is an essential chaperone that is specific for collagen maturation and an increase in α-SMA is indicative of the activation of HSCs. The SOD1 KO mice exhibited higher HSP47 protein levels although the SOD1 KO and SOD1 Tg mice livers both showed higher levels of α-SMA (Figure 2C).

**α-SMA and HSP47 were highly expressed in HSCs of SOD1 KO mice livers**

Immunohistochemistry was carried out to determine the localization of α-SMA, and HSP47 in mice livers. α-SMA proteins were highly expressed in fibrosis areas of SOD1 KO mice livers (Figure 3, upper panel). SOD1 Tg mice livers also slightly express α-SMA proteins, which appear to be matched with the protein levels (Figure 2C). In contrast, HSP47 proteins were expressed near the fibrosis areas of only SOD1 KO mice livers (Figure 3, middle panel). These results show that the accumulation of collagen, which was caused by an SOD1 deficiency were correlated with the activation of α-SMA and HSP47 in
HSCs. In addition, iron accumulation in liver would progress hepatic fibrosis. Therefore, the liver iron levels were compared among WT, SOD1 KO and Tg mice. As shown in Figure 3 (lower panel), some iron deposits were detected in SOD1 KO mice livers but not in both WT and SOD1 Tg mice livers.

**SOD1 Tg mice livers possess higher MMP9 activity**

Next, to investigate collagen degradation activity in these mice livers, gelatin zymography was carried out. Unexpectedly, the zymography showed that MMP2 and MMP9 activities were increased in both SOD1 KO and Tg mice, and that MMP9 activity was measurably higher in both mice (Figure 4A). However, this MMP activity, as measured by zymography, reflects both active-MMP and inactive-MMP, which is associated with TIMPs. Thus, we found substantial MMPs enzymatic activities in mice livers by a FRET peptide assay under conditions of the absence of APMA, an activator of pro-MMPs. As shown in Figure 4(B), the MMP9 activity in SOD1 Tg mice livers was much higher than those for the other mice, as
expected. These results suggest that excessive collagen could be cleaved by MMP2 or MMP9 in SOD1 Tg mice, and therefore no accumulation of collagen would be observed in SOD1 Tg mice livers.

MMP activity is inhibited by TIMP1 in SOD1 KO mice livers

To confirm the decrease in MMP9 activity in SOD1 KO mice livers, the mRNA levels of MMP2, 9, and TIMP1, 2, 3, 4 were measured by semi qRT-PCR. As shown in Figure 5(A), the gene expressions of MMP2 and MMP9 in both SOD1 KO and Tg mice were higher than the corresponding values for WT mice. In addition, although there was no change in the mRNA levels of TIMP2, 3 and 4, TIMP1 mRNA was increased only in SOD1 KO mice livers. As expected, although MMP9 proteins were highly expressed in both SOD1 KO and Tg mice, higher levels of TIMP1 were expressed only in SOD1 KO mice livers (Figure 5B), which indicates that the increased TIMP1 inhibits MMP9 activity in SOD1 KO mice livers. To clear how SOD1 deficiency cause increased TIMP1, MMP9, and HSP47 expression, HepG2 cells were treated with DMNQ (superoxide generator) and H₂O₂, and then the mRNA levels of these proteins were estimated by RT-PCR. As shown in Supplementary Figure S2, TIMP1 mRNA levels were upregulated by the treatment with DMNQ or H₂O₂ and HSP47 was induced by the treatment of H₂O₂. Therefore, we consider that TIMP1 induced by large amount of superoxide and following
produced H₂O₂ by spontaneous reaction by SOD1 deficiency would inhibit degradation of collagen in SOD1 KO mice livers.

**SOD1 KO mice liver proteins are modified with AGEs**

SOD1 KO mice show higher blood glucose levels (Figure 6A, [35]). We therefore hypothesized that oxidative stress and the higher blood glucose concentration in SOD1 mice livers would enhance the modification of collagen by AGEs, and that this would interrupt the degradation of collagen in SOD1 mice livers. Typical AGE components, such as CMA and CML, are thought to be one of the useful markers of oxidative damage [36]. In addition, increases in AGEs such as CMA and CML would lead to the expression of a receptor of AGE (RAGE) [37,38]. Thus, we estimated AGE levels by immunohistochemical staining using an anti-CMA and anti-CML antibodies on serial liver sections and measured the expression level of RAGE. As shown in Figure 6(B), a large number of CMA and CML immunospots were observed only in SOD1 KO mice livers. The expression of RAGE was also significantly increased both in SOD1 KO and Tg mice livers with both mRNA and protein levels (Figure 6C).

**Discussion**

In the present study, we demonstrated the progression from fatty liver to fibrosis in SOD1 KO mice, even when they were fed a normal diet. Although some lipid droplets and higher expression levels of α-SMA proteins were observed, no accumulation in collagen was found.
in the livers of SOD1 Tg mice (Figures 1 and 2). In contrast, not only higher expression levels of the collagen chaperon, HSP47 (Figure 2C), but also higher expression levels of the collagen protease inhibitor, TIMP1 (Figure 5), were detected only in SOD1 KO mice livers. In addition, immunohistochemical analyses revealed that α-SMA and HSP47 proteins were highly expressed in fibrosis areas of SOD1 KO mice livers (Figure 3). These findings provide an explanation for why collagen accumulates in SOD1 KO mice livers. The expression of HSP47 in various cell lines and tissues is closely correlated with the expression levels of various types of collagen molecules [39–43]. In fact, we observed that HSP47 levels were increased prior to the onset of NASH in the livers of a NASH-HCC (STAM® mice) model mouse (data not shown). Collagen producing cells, HSC/myofibroblasts, have been reported to show a marked upregulation of TIMP1 during activation, proceeding collagen expression, and potently inhibit MMP activity [44,45]. Elevated levels of TIMP1 have also been observed

Figure 6. The effect of glycation on collagen accumulation. (A) The blood glucose level of WT (10–12 weeks), SOD1 KO (10–12 weeks), and SOD1 Tg (10–12 weeks) mice. (B) Immunohistochemistry for CMA and CML in liver section of WT (10–12 weeks), SOD1 KO (10–12 weeks), and SOD1 Tg (10–12 weeks) mice. The horizontal black bar indicates 50 μm. (C) The mRNA expression levels of RAGE in WT, SOD1 KO, and SOD1 Tg mice livers at 10–12 weeks by RT-PCR (a). The protein expression levels of RAGE in WT, SOD1 KO, and SOD1 Tg mice livers at 10–12 weeks by Western blotting (b). The bottom graph shows the fold changes calculated against the protein levels of WT mice by the densitometric scanning of western blots. The data are normalized to the expression levels of β-actin and expressed as means ± SE (*p < 0.05).
during progressive fibrosis in humans and experimental model animals [46,47]. The critical role of TIMP1 in fibrogenesis and resolution was confirmed using a transgenic system, whereby hepatic TIMP1 overexpression accelerated fibrogenesis but also caused a failure of scar resolution [48]. Therefore, TIMP1 would be expected to be a key regulator of the progression of liver fibrosis. A series of strategies have been explored in experimental fibrosis, including myofibroblast activation, myofibroblast apoptosis and a MMP/TIMP imbalance [49,50]. A cure that targets TIMP1 may represent a useful strategy in the future. Therefore, we conclude that the enhanced production of HSP47 and TIMP1 as the result of oxidative stress is responsible for the progression of hepatic fibrosis.

Then, why will MMPs and TIMP elevated in SOD1 KO mice livers? The mRNA levels of TIMP1 were upregulated by treatment of DMNQ and H2O2 and HSP47 were upregulated by H2O2 in HepG2 (Supplementary Figure S2). From this result, TIMP1 which is induced by large amount of superoxide and following produced H2O2 due to SOD1 deficiency inhibits degradation of collagen and then accumulates collagen in SOD1 KO mice livers.

Iron overload would enhance oxidative stress. Since SOD1 KO mice exhibit hemolytic anemia [22], the excess iron from the destroyed erythrocytes would induce glutathione S-transferase A4 (GSTA4) in kidneys but not in livers [23]. The difference between normal kidneys and abnormal livers observed in SOD1 KO mice might be due to the compensatory induction of other anti-oxidative enzymes like GSTA4. The resultant iron deposits detected in SOD1 KO mice livers (Figure 3, [29,31]) would lead to further progression of hepatic fibrosis and carcinoma. However, SOD1 deficiency and high fat diet synergistically enhanced hepatic lipid accumulation in mice [26]. Based on this result, high fat diet condition also may accumulate hepatic collagen and liver fibrosis in SOD1 KO mice.

SOD1 KO mice exhibit higher blood glucose levels (Figure 6A, [35]), although the blood glucose levels in SOD1 Tg mice were almost same as those of WT mice. Because of this, RAGE expression and AGEs accumulation are increased in SOD1 KO mice livers. Higher AGE levels in the serum or liver were reported in patients with steatohepatitis compared to healthy controls or patients with simple steatosis [51]. RAGE expression was also significantly increased in patients with NASH [52]. Because collagen is a relatively stable protein, it could easily become a target for various types of post-translational modifications such as glycation (AGE modification). Some AGEs involve the formation of a bridge between free amino groups of neighboring proteins to form cross-links. Among the AGEs, one that is specific to collagen tissues has recently been reported to be CMA. CML is also one of AGEs and is formed by oxidative cleavage of Amadori products [53–55] CML and CMA immunospots were observed only in SOD1 KO mice livers (Figure 6B). The modification of liver proteins including collagen by AGEs would be one of the reasons for why collagen is not easily cleaved by MMPs in SOD1 KO mice livers.

In conclusion, the findings reported herein demonstrate that an imbalance in MMP/TIMP and an excess accumulated collagen are the cause of the fibrosis in KO mice livers, strongly indicating that oxidative stress leads to hepatic fibrosis. Although further studies will be required to clarify the molecular mechanism of upregulation TIMP1 and AGE modification by oxidative stress, the downregulation of TIMP1 and/or AGE modification represent a potential cure for liver fibrosis.

Disclosure statement
The authors declare that they have no conflicts of interest.

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See <http://journalauthors.tandf.co.uk/preparation/multimedia.asp> for further explanation of supplemental data and underlying research materials.

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