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Article

Smad3 Deficiency Ameliorates Hepatic Fibrogenesis through the Expression of Senescence Marker Protein-30, an Antioxidant-Related Protein

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Abstract: Smad3 is a key mediator of the transforming growth factor (TGF)-β1 signaling pathway that plays central role in inflammation and fibrosis. In present study, we evaluated the effect of Smad3 deficiency in Smad3−/− mice with carbon tetrachloride (CCL4)-induced liver fibrosis. The animals were received CCL4 or olive oil three times a week for 4 weeks. Histopathological analyses were performed to evaluate the fibrosis development in the mice. Alteration of protein expression controlled by Smad3 was examined using a proteomic analysis. CCL4-induced liver fibrosis was rarely detected in Smad3−/− mice compared to Smad3+/+. Proteomic analysis revealed that proteins related to antioxidant activities such as senescence marker protein-30 (SMP30), selenium-binding proteins (SP56) and glutathione S-transferases (GSTs) were up-regulated in Smad3−/− mice. Western blot analysis confirmed that SMP30 protein expression was increased in Smad3−/− mice. And SMP30 levels were decreased in CCL4-treated Smad3+/+ and Smad3−/− mice. These results indicate that Smad3 deficiency influences the proteins level related to...
antioxidant activities during early liver fibrosis. Thus, we suggest that Smad3 deteriorate hepatic injury by inhibitor of antioxidant proteins as well as mediator of TGF-β1 signaling.

**Keywords:** liver fibrosis; Smad3; SMP-30; antioxidant

1. Introduction

Smad proteins are intracellular mediators that respond to transforming growth factor (TGF)-β, an important regulatory cytokine that affects the production, degradation, and accumulation of extracellular matrix proteins during the development of liver fibrosis [1]. Smad proteins are involved in intracellular signaling in response to TGF-β family members. Smad proteins are classified as receptor-regulated Smads (R-Smads), common mediator Smads (Co-Smads), or inhibitory Smads (I-Smads). R-Smads are directly phosphorylated by receptors and translocate to the nucleus where they bind to response elements and regulate gene expression [2]. Smad3 belongs to the R-Smads family. This factor is phosphorylated by receptors and translocates to the nucleus where it activates gene expression.

Impairment of the Smad pathway results in an escape from growth inhibition, thereby promoting cell proliferation and contributing to carcinogenesis [3]. In particular, Smad3 is a major player in signal transduction pathways associated with fibrogenesis [4]. And several fibrotic markers are attenuated in Smad3 knockout (KO) mice, resulting in the hypothesis that directing Smad3 to a clinical target might inhibit fibrosis [5,6]. In addition, the radiation-induced expression of α-smooth muscle actin (α-SMA) in cutaneous fibroblasts of Smad3 KO mice is reduced to 25% of that found in cells from Smad3 wild-type (WT) mice [7,8]. When acute liver injury is induced by the administration of CCl4, Smad3 KO mice show approximately one-half of the induction of hepatic collagen type I mRNA expression compared to Smad3 wild type (WT) mice [9]. These results have led to the speculation that Smad3 signaling specifically mediates the fibrotic effects of TGF-β. But, previous studies of Smad3 targets in an animal model of liver injury did not reveal a clear-cut mechanism underlying such activity.

A hepatic proteomic analysis was also conducted to study thioacetamide-induced cirrhosis in rats [10]. However, no proteomic analysis has unequivocally determined the relationship between Smad3 and hepatic fibrogenesis.

Here, we investigated the influence of Smad3 on liver fibrogenesis by measuring hepatic protein expression in Smad3 KO mice after CCl4 treatment for 4 weeks. Histological and proteomic analyses were used to monitor changes of protein expression during the course of hepatic fibrogenesis in these mice.

2. Results

2.1. Fibrotic Changes in Livers after the Chronic Administration of CCl4

To evaluate the influence of Smad3 deficiency on hepatic fibrogenesis induced by repeated CCl4 injection, we first examined the H & E-stained livers of Smad3 WT and Smad3 KO mice (Figure 1A,B). Histological analysis revealed an increased hepatocyte population and loosening of the cell-to-cell tight junctions in the Smad3 KO mice. When sections from the Smad3 KO mice were
assessed with immunohistochemical staining using an anti-PCNA antibody (Figure 1D), hepatocyte replication in the livers of Smad3 KO mice was found to be markedly greater than that found in Smad3 WT mice (Figure 1C).

**Figure 1.** Histopathological analysis of livers from Smad3$^{+/+}$ and Smad3$^{-/-}$ mice. Liver tissues from Smad3$^{+/+}$ (A and C) and Smad3$^{-/-}$ (B and D) mice. Results for H & E staining (A and B) and immunohistochemistry for PCNA (C and D) are shown. Scale bars represent 50 µm. Inserts are high magnification fields from each figure. Scale bars represent 100 µm.

**Figure 2.** Hepatic fibrotic changes of Smad3 mutant mice after CCl$_4$ treatment. Liver tissues of CCl$_4$-treated Smad3$^{+/+}$ (A, C and E) and Smad3$^{-/-}$ (B, D and F) mice. H & E stain (A and B); azan stain (C and D); and immunostain for α-SMA (E and F). Scale bars represent 25 µm. Inserts (A and B) are von Kossa-stained samples of each figure. Scale bars represent 100 µm.
After four weeks of exposure to CCl₄, liver fibrosis in the Smad3 WT mice was more severe than in the Smad3 KO mice (Figure 2 and Table 1). However, more calcium deposition was detected in the CCl₄-treated Smad3 KO mice compared to the CCl₄-treated Smad3 WT mice (inserts of Figure 2A,B). These findings were observed focally in the centrilobular region and destroyed hepatocytes of the CCl₄-treated Smad3 KO mice. The fibrotic changes were confirmed in liver tissues stained with azan (Figure 2C,D). Additionally, immunohistochemical analysis revealed that the expression of α-SMA in the livers of CCl₄-treated Smad3 WT mice was greater than that in the Smad3 KO mice (Figure 2E,F and Table 1).

### Table 1. Hepatic lesions and grade of fibrosis in the livers of Smad3-mutant mice after the CCl₄-treatment.

<table>
<thead>
<tr>
<th>Animal treatment (number of animals)</th>
<th>Hepatic lesions after CCl₄-treatment</th>
<th>Grade A</th>
<th>α-SMA B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smad3&lt;sup&gt;WT&lt;/sup&gt; mice (&lt;i&gt;n&lt;/i&gt; = 5)</td>
<td>Normal</td>
<td>0.10 ± 0.22</td>
<td>0.74 ± 0.25</td>
</tr>
<tr>
<td>Smad3&lt;sup&gt;KO&lt;/sup&gt; mice (&lt;i&gt;n&lt;/i&gt; = 5)</td>
<td>Enlargement of hepatocyte</td>
<td>0.08 ± 0.18</td>
<td>0.68 ± 0.22</td>
</tr>
<tr>
<td>CCl₄-treated Smad3&lt;sup&gt;WT&lt;/sup&gt; mice (&lt;i&gt;n&lt;/i&gt; = 5)</td>
<td>Pseudolobule formation, Degenerative and necrotic cells</td>
<td>2.60 ± 0.47 *</td>
<td>2.40 ± 0.47 *</td>
</tr>
<tr>
<td>CCl₄-treated Smad3&lt;sup&gt;KO&lt;/sup&gt; mice (&lt;i&gt;n&lt;/i&gt; = 5)</td>
<td>Bridging fibrosis, Severe calcium deposition</td>
<td>1.40 ± 0.55 *</td>
<td>1.84 ± 0.49 *</td>
</tr>
</tbody>
</table>

<sup>A</sup> Grade of hepatic fibrosis—0, none; 1, short collagenous septa extended from central veins; 2, slender septa link the central veins but lobular architecture is preserved; 3, pseudolobuli are formed by thin septa; 4, parenchyma is subdivided into smaller pseudolobuli by thin septa. Liver fibrosis was graded based on a previously reported scoring system [11]; <sup>B</sup> Immunohistochemical expression for α-smooth muscle actin—0, none; 1, mild; 2, moderate; 3, severe expression. Data are shown in Figure 2C; * Significant differences in Smad3<sup>WT</sup> and Smad3<sup>KO</sup> mice (Student’s <i>t</i> test, <i>p</i> value < 0.005).

### 2.2. Analysis of Smad3-Related Proteins in Liver after the Chronic Administration of CCl₄

To investigate possible differences of protein expression in the livers of Smad3 WT and Smad3 KO mice, the overall protein content in liver tissues of both groups was analyzed by using two-dimensional gel (2-DE) electrophoresis (Figure 3A) and mass spectrometry (MS). As a result, we found that ten proteins were down-regulated and eight proteins were up-regulated in the Smad3 KO mice compared to the Smad3 WT mice after four weeks of exposure to CCl₄ treatment (Table 2). As shown in Figure 4A, glutathione peroxidase, glutathione S-transferase theta 2 (GST class-theta 2), NADH-ubiquinone oxidoreductase (subunit B14.7) and peroxiredoxin 6 (antioxidant protein 2) were significantly increased in CCl₄ treated Smad3 KO mice. These proteins play an important role in the defense against oxidative stress by reducing reactive oxygen species (ROS). Next, we confirmed the proteomic result by western blotting. SMP30 was increased in CCl₄ treated Smad3 KO mice, compared to CCl₄ treated Smad3 WT mice (Figure 3B). Also, SMP30 protein level was elevated in control Smad3 KO mice, compared to control Smad3 WT mice (Figure 3C).
Figure 3. Substantial differentiated spots in liver tissues of Smad3−/− mice. (A) Gel images of two dimensional gel electrophoresis (2-DE). Protein from liver tissue of Smad3+/+ and Smad3−/− mice treated with CCl4 was separated on a pH 3–10 IPG strip in the first dimension and on an SDS-PAGE (12%) gel in the second dimension; (B) Clearly visible spots of the four proteins HSP70, SMP30, SP56 and GST are reproduced from gel images (SDS-PAGE) on a zoom-in view. WT: Smad3+/+ mice treated with CCl4; KO: Smad3−/− mice treated with CCl4; and (C) Immunoblot analysis for SMP30 and β-actin (as an internal standard).

Table 2. List of notably changed proteins in Smad3 KO mice liver after CCl4 treatment.

<table>
<thead>
<tr>
<th>Spot No.</th>
<th>Protein Name</th>
<th>Accession No. a</th>
<th>MOWSE Score b</th>
<th>Masses Matched (%)</th>
<th>MW (kDa)</th>
<th>pI c</th>
<th>Relative ratios d</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Down-regulated proteins in CCl4-treated KO mice compared to CCl4-treated WT mice</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>54</td>
<td>Rho guanine nucleotide exchange factor 7</td>
<td>Q9ES28</td>
<td>3.32 × 10⁶</td>
<td>17</td>
<td>80</td>
<td>5.50</td>
<td>0.30</td>
</tr>
<tr>
<td>55</td>
<td>78 kDa glucose-regulated protein precursor</td>
<td>P20029</td>
<td>1.48 × 10⁷</td>
<td>36</td>
<td>72</td>
<td>5.10</td>
<td>0.21</td>
</tr>
<tr>
<td>56</td>
<td>Heat shock cognate 71 kDa protein (HSP 70)</td>
<td>P63017</td>
<td>7.85 × 10⁹</td>
<td>44</td>
<td>70</td>
<td>5.40</td>
<td>0.36 *</td>
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<tr>
<td>57</td>
<td>Leucine-rich repeat LGI family member 4 precursor</td>
<td>Q8K1S1</td>
<td>1.46 × 10⁴</td>
<td>10</td>
<td>59</td>
<td>7.90</td>
<td>0.19</td>
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<tr>
<td>59</td>
<td>Adenosylhomocysteinase (Liver copper binding protein)</td>
<td>P50247</td>
<td>7.03 × 10⁹</td>
<td>13</td>
<td>48</td>
<td>5.10</td>
<td>0.18</td>
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<tr>
<td>60</td>
<td>Neutrophil cytosol factor 1 (NCF-1)</td>
<td>Q09014</td>
<td>1.25 × 10⁶</td>
<td>12</td>
<td>45</td>
<td>9.10</td>
<td>0.15</td>
</tr>
<tr>
<td>61</td>
<td>Wnt-11 protein precursor</td>
<td>P48615</td>
<td>1.77 × 10⁷</td>
<td>15</td>
<td>39</td>
<td>9.10</td>
<td>0.27</td>
</tr>
<tr>
<td>62</td>
<td>Uricase (Urate oxidase)</td>
<td>P35688</td>
<td>1.33 × 10⁶</td>
<td>21</td>
<td>35</td>
<td>8.50</td>
<td>0.24</td>
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<tr>
<td>63</td>
<td>Thioether S-methyltransferase (TEMT)</td>
<td>P40936</td>
<td>1.96 × 10⁵</td>
<td>15</td>
<td>30</td>
<td>6.00</td>
<td>0.43</td>
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<tr>
<td>64</td>
<td>Ferritin light chain 1 (Ferritin L subunit 1)</td>
<td>P29391</td>
<td>9.28 × 10⁵</td>
<td>22</td>
<td>21</td>
<td>5.70</td>
<td></td>
</tr>
</tbody>
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Table 2. Cont.

<table>
<thead>
<tr>
<th>Spot No.</th>
<th>Protein Name</th>
<th>Accession No. a</th>
<th>MOWSE Score b</th>
<th>Masses Matched (%)</th>
<th>MW (kDa)</th>
<th>pI c</th>
<th>Relative ratios d</th>
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<tr>
<td>19</td>
<td>Selenium-binding protein 1 (SP56)</td>
<td>P17563</td>
<td>1.24 × 10^4</td>
<td>18</td>
<td>52</td>
<td>6.00</td>
<td>2.86 *</td>
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<tr>
<td>21</td>
<td>Senescence marker protein-30 (SMP30)</td>
<td>Q64374</td>
<td>3.55 × 10^6</td>
<td>22</td>
<td>33</td>
<td>5.20</td>
<td>2.58 *</td>
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<tr>
<td>22, 23</td>
<td>Glutathione S-transferase Mu 1</td>
<td>P10649</td>
<td>1.29 × 10^6</td>
<td>21</td>
<td>26</td>
<td>7.70</td>
<td>2.61 *</td>
</tr>
<tr>
<td>65</td>
<td>Glutathione peroxidase</td>
<td>P11352</td>
<td>8.77 × 10^4</td>
<td>10</td>
<td>22</td>
<td>6.70</td>
<td>5.07</td>
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<tr>
<td></td>
<td>(Cellular glutathione peroxidase)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>66</td>
<td>Glutathione S-transferase theta 2</td>
<td>Q61133</td>
<td>1.12 × 10^4</td>
<td>7</td>
<td>28</td>
<td>7.00</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(GST class-theta 2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>67</td>
<td>NADH-ubiquinone oxidoreductase subunit B14.7</td>
<td>Q9D8B4</td>
<td>4.40 × 10^4</td>
<td>10</td>
<td>15</td>
<td>8.60</td>
<td>12.15</td>
</tr>
<tr>
<td>68</td>
<td>Peroxiredoxin 6</td>
<td>O08799</td>
<td>5.65 × 10^4</td>
<td>19</td>
<td>25</td>
<td>5.70</td>
<td></td>
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<tr>
<td>69</td>
<td>Alpha-1-acid glycoprotein 1 precursor</td>
<td>Q60590</td>
<td>2.60 × 10^4</td>
<td>10</td>
<td>24</td>
<td>5.60</td>
<td>2.39</td>
</tr>
</tbody>
</table>

a Accession No.: Protein No. of SwissProt database; b MOWSE score: Based on the number of peptides matching the protein in the database and the accuracy of those matches; c MW and pI: Obtained from the MS fit search of proteinprospector database; d Relative ratio: Relative % volume of spot in KO compared to WT (↑, up-regulation in knockout type; ↓, down-regulation in knockout type); * Significant differences in the Smad3 WT mice (Student’s t test, p value < 0.05).

Figure 4. Expression of p-Smad3 in the SMP30Y−/− mice. Immunohistopathological detection of p-Smad3 in liver tissues from (A) SMP30Y+/−; (B) CCl4-treated SMP30Y+/−; (C) SMP30Y−/−; and (D) CCl4-treated SMP30Y−/− mice. Cont indicates the non-treated control. Expression of p-Smad3 in the CCl4-treated liver significantly increased around the central vein in SMP30Y−/− mice (D) compared to SMP30Y+/− mice (B). Scale bars = 100 µm.

2.3. Increased Expression of Smad3 in SMP30 KO Mice

To confirm the relationship between Smad3 and SMP30 in vivo, we used SMP30 KO (SMP30Y−/−) mice and analyzed phospho-Smad3 (p-Smad3) levels in their livers acutely damaged by CCl4 treatment. Immunohistochemical examination revealed that an exposure to CCl4 increased p-Smad3 levels in the livers of SMP30 KO mice as well as WT mice; however, the SMP30 KO mice had significantly more p-Smad3 than the SMP30 WT mice (Figure 4).
3. Discussion

A major finding of the present study is that Smad3 deficiency significantly prevented CCl4-induced liver fibrosis by reducing antioxidant protein expression as well as blocking TGF-β signaling in the liver. Treatment of Smad3 KO mice with CCl4 for 4 weeks successfully induced moderate liver fibrosis. Findings from the histological analysis correlated with data from immunohistochemical testing in which α-SMA was used as a fibrotic marker. All of these data suggested that the absence of Smad3 resulted in reduced hepatic damage due to CCl4 treatment. On the other hand, high levels of calcium deposition were observed in the centrilobular areas of Smad3 KO mice, indicating that the hepatocytes had sustained severe damage. Haschek and Rousseaux demonstrated that increased intracellular calcium contents exacerbate cellular damage, resulting in hepatic fibrosis [12]. Interestingly, CCl4-treated Smad3 KO mice in the present study had more calcium deposition than CCl4-treated WT animals, but less fibrosis was found in the CCl4-treated Smad3 KO mice. This is not consistent with findings from the study by Haschek and Rousseaux. We questioned why Smad3 deficiency resulted in less fibrosis even with significant calcium deposition. So far, we have not identified a clear reason for this result. We assumed that the high levels of calcium deposition in CCl4-treated Smad3 KO mice may not be related to severe cell damage because 1) an increase in PCNA-positive signals (usually detected during active cell proliferation) was found in the CCl4-treated Smad3 KO mice, and 2) up-regulation of cell protective gene expression was identified during proteomic analysis.

Proteomic evaluation demonstrated that CCl4-treated Smad3 KO mice had increased expression of antioxidant-related proteins such as SMP30, SP56, and GST. In particular, SMP30 expression was markedly up-regulated in Smad3 KO mice (Table S1), compared to WT mice. SMP30 is a major senescence marker and its expression is reduced during the aging process [13], SMP30 is also involved in the vitamin C synthesis pathway; SMP30 KO mice cannot synthesize vitamin C in vivo [14]. In a recent report, Son et al. demonstrated that SMP30 exerts a potent anti-oxidative effect and protects neural cells from oxidative damage [15]. Thus, we assumed that deletion of the Smad3 protein triggered an increase of SMP30 protein expression, resulting in the prevention of hepatic cell death due to CCl4-induced injury. Although further studies are required to determine whether SMP30 is a key protein that directly prevented liver fibrosis in the present study, the fact that Smad3 deficiency led to up-regulated SMP30 expression supports the speculation that SMP30 expression may play an opposing role to that of Smad3 during the induction of hepatic fibrosis through an unknown mechanism.

The phenomenon was confirmed by another set of animal experiments with SMP30 KO mice, indicating that SMP30 deficiency increases the levels of phospho-Smad3 which is a key protein to promote liver fibrosis. To investigate the precise role of SMP30, we evaluated male SMP30 KO mice with acute CCl4-induced hepatotoxicity. After CCl4-induced injury, the levels of phospho-Smad3 in the SMP30 KO mice were significantly higher than those in the SMP30 WT littermates. Enhanced expression of phospho-Smad3 in the SMP30 KO mice we observed concurred with our finding of decreased hepatic fibrogenesis in Smad3 KO mice. In general, the phosphorylation of Smad3, a key modulator of the TGF-β1 signaling pathway, is induced during hepatic fibrogenesis [2].

The expression of glutathione peroxidase, GST theta, NADH-ubiquinone oxidoreductase, and peroxiredoxin 6 (antioxidant protein 2) was also up-regulated in Smad3 KO mice (data not shown).
These proteins are involved in metabolism and are associated with the ROS defense systems [16]. In particular, peroxiredoxin 6 has been known as an acidic calcium-independent phospholipase A2 and a non-selenium glutathione peroxidase. Since proteins related to the antioxidant system were more highly expressed in Smad3 KO mice than in the Smad3 WT mice after CCl4 treatment, their presence presumably provided a significant level of protection against hepatic fibrosis.

4. Material and Methods

4.1. Animals

Smad3 KO mice generated by targeted disruption of the Smad3 gene via homologous recombination were kindly provided by Anita B Robert (National Cancer Institute, Bethesda, MD, USA). These mutant mice were housed in a room at 22 ± 2 °C, with a 12-h light-dark cycle and were given food and water ad libitum. Genotypes of Smad3 mutant mice were determined using tail DNA (Figure S1A). For PCR analysis, the Smad3 WT allele was detected using primer 1: 5'-CCACTTCATTGCCATATGCCCTG-3' and primer 2: 5'-CCCGAACAGTTGGATTTCACACA-3'. This primer pair amplifies a fragment of 431 bp from Smad3 WT mice, but not from Smad3 KO mice. DNA was also amplified using the primer 1 and primer 3: 5'-CCAGACTGCCTTGGGAAAAGC-3', which is located in the pLoxneo to detect the mutant Smad3 allele. In this case, a 284 bp fragment was detected for the Smad3 KO allele, whereas no such signal was detected in the wild-type mice [17]. Smad3 KO mice were previously generated with the gene targeting technique and kindly provided by Akihito Ishigami in Tokyo Metropolitan Institute of Gerontology (Tokyo, Japan) and heterozygous female mice (SMP30+/−) were mated with male knockout mice (SMP30Y/−) to produce male knockout (SMP30Y/−) and male wild type (SMP30Y+/+) littermates. Genotypes of SMP30 mutant mice were determined as described previously (Figure S1B) [18].

4.2. Treatment of Animals

Six-week-old male Smad3 WT and Smad3 KO mice were divided into four groups: Smad3 WT, Smad3 KO, CCl4-treated Smad3 WT, and CCl4-treated Smad3 KO mice. In the CCl4-treated animals, liver fibrosis was induced by intraperitoneal injection of 2 mL/kg of CCl4 (Wako Pure Chemical Industries, Osaka, Japan) three times a week for 4 weeks. The mice were sacrificed 24 h after the last injection. For the induction of acute liver injury, SMP30Y/+ and SMP30Y/− mice were injected intraperitoneally with CCl4 every 2 days for a week. Twenty-four hours after the third CCl4 injection, liver tissues were collected from all mice.

4.3. Histopathological Evaluation of the Liver Tissue

Pieces of liver were rapidly fixed in 10% neutral buffered formalin and embedded in paraffin. The sections were stained with hematoxylin and eosin (H & E), and azan. The severity of hepatic fibrosis was graded on a scale of 0–4 as previously reported [11]. Calcium deposition in liver tissues from the CCl4-treated groups was evaluated using von Kossa stain. For immunohistochemistry, anti-PCNA (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-α-SMA (Sigma, St. Louis, MO, USA), and anti-phosphorylated (phospho)-Smad3 (Cell Signaling Technology, Danvers, MA, USA) antibodies
were used as primary antibodies. Antigen-antibody complexes were visualized by using an avidin-biotin peroxidase complex solution and an ABC kit (Vector Laboratories, Burlingame, CA, USA) with 3,3-diamino benzidine (Zymed Laboratories Inc., South San Francisco, CA, USA).

4.4. Preparation of Liver Tissue for Proteomic Analysis

Frozen liver tissue (200 mg) was homogenized in lysis buffer (40 mM Tris, 8 M urea, 4% CHAPS, 1 mM EDTA, and 10 mM dithioerythritol). The protein concentration of the homogenates was estimated using a commercial 2-D Quant Kit (Amersham Biosciences, Uppsala, Sweden) [19].

4.5. Two-Dimensional Gel Electrophoresis and Data Processing

A first-dimensional isoelectric focusing (IEF) experiment was carried out on commercial 18 cm, immobilized pH gradient (IPG) strips (pH 3–10), in an IPGphor electrophoretic unit (Amersham Biosciences, Pittsburgh, PA, USA). The second-dimension was separated on 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) at 15 °C in an Ettan DALTs six apparatus (Amersham Biosciences, Pittsburgh, PA, USA). All samples were run in triplicate to ensure reproducibility of results. After fixation, the gels were stained with Coomassie blue (Bio Basic Inc., Markham, ON, Canada). Spot intensities in gel image were analyzed with PDQuest 2D Analysis Software (BioRad, Hercules, CA, USA). Statistical analysis of the spot intensities was performed by Student’s t-test ($p < 0.05$). The significance of level was taken more than two-fold changes of volume percentage [20].


Targeted spots were excised, and an in-gel digestion was performed. Briefly, enzymatic digestion was performed by adding modified trypsin (Promega, Madison, WI, USA) in 50 mM ammonium bicarbonate and 5 mM calcium chloride then incubation at 37 °C for 16 h. These spots were analyzed by using MALDI-TOF/MS (Voyager-DE STR, Applied Biosystems, Foster, CA, USA). A database search was then conducted with the MS-Fit (http://prospector.ucsf.edu) to locate possible matches.

4.7. Immunoblotting

Snap-frozen liver tissues were homogenized in a RIPA buffer containing 0.1 mM of Na$_3$VO$_4$ and Protease Inhibitor Cocktail Tablets (Roche, Mannheim, Germany). Protein samples (30 µg per lane) were separated by 10% SDS-PAGE and electro-transferred to a PVDF membrane (Schleicher & Schuell, Dassel, Germany). The primary antibodies used were anti-SMP30 antibody (SantaCruz Biothechnology, Dallas, TX, USA), and anti-β-actin antibody (SantaCruz Biotechnology, Dallas, TX, USA). Specific binding was detected by using the Super Signal West Dura Extended Duration Substrate (Pierce, Rockford, IL, USA) and by exposure of the blots to Medical X-ray Film (Kodak, Tokyo, Japan).

4.8. Statistical Analysis

The results are expressed as the means ± S.D. To compare values obtained from the two groups, Student $t$ test was done. A value of $p < 0.05$ was considered significant.
5. Conclusions

Results from the current investigation indicated that Smad3 likely participates in the antioxidant defense system by regulating the production of antioxidant proteins such as SMP30 that protects against liver injury and mediates TGF-β signaling. A more depth-in study will be required to further elucidate the underlying mechanism. Based on our findings, we suggest that the Smad3 may play a critical role in hepatic fibrosis development mediated by the regulation of antioxidant gene expression and TGF-β signaling.

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Conflicts of Interest

The authors declare no conflict of interest.

References


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