Zeaxanthin Dipalmitate from Lycium chinense Fruit Reduces Experimentally Induced Hepatic Fibrosis in Rats

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We previously reported that zeaxanthin dipalmitate (ZD), a carotenoid from Lycium chinense fruit, reduces myofibroblast-like cell proliferation and collagen synthesis in vitro. To determine whether ZD might reduce the severity of hepatic fibrosis in an animal model, hepatic fibrosis was induced in rats by bile duct ligation/scission (BDL) for a period of 6 weeks. Treatment of BDL rats with ZD at a dose of 25 mg/kg body weight significantly reduced the activities of aspartate transaminase (p<0.05) and alkaline phosphatase (p<0.001) in serum. Furthermore, collagen deposition was significantly reduced as assessed by the Sirius Red binding assay in BDL rats administered ZD at the dose of 25 mg/kg body weight (p<0.01). In addition, the levels of thioibarbituric acid-reactive substances and 4-hydroxyproline were reduced when BDL rats received ZD at the dose of 25 mg/kg body weight. These results showed that ZD effectively inhibited hepatic fibrosis in BDL rats, at least in part via its antioxidative activity.

Key words zeaxanthin dipalmitate; Lycium chinense; bile duct ligation; antifibrotic activity; antioxidative activity

Hepatic fibrosis is a common response to chronic liver injury due to ethanol consumption, viral infection, oxidative stress, and biliary disorders. During fibrogenesis, hepatic stellate cells (HSCs), which are a major source of extracellular matrix in both normal and pathological conditions, undergo a sequential process of transactivation, thereby developing a myofibroblastic phenotype associated with increased proliferation and collagen synthesis.

The dried ripe fruits of Lycium chinense have been used as a tonic in Oriental medicine, and are believed to have antihypertensive activity, inhibitory effects on the development of fatty liver, and the ability to reduce the content of sugar in the blood. We previously reported that zeaxanthin dipalmitate (ZD, Fig. 1), a carotenoid from L. chinense fruits, significantly protected primary cultures of rat hepatocytes against carbon tetrachloride (CCl4)-induced toxicity and reduced the proliferation of myofibroblast-like cell (MFBLC) and collagen synthesis in cultured HSCs in vitro.

The current study was designed to evaluate whether ZD exhibited antifibrotic activity in an animal model of hepatic fibrosis such as bile duct ligation/scission (BDL) rats since it showed protective activity in vitro.

MATERIALS AND METHODS

Animal Model of Liver Fibrosis Female Wistar rats (200±20 g) were obtained from the Laboratory Animal Center, Seoul National University. They were fed on standard rat chow with free access to tap water, and kept in temperature- and humidity-controlled animal quarters under a 12-h light–dark cycle. All experiments were conducted according to the guidelines of the Committee on Care and Use of Laboratory Animals of Seoul National University. Secondary biliary fibrosis was induced by BDL as described previously. Sham-operated animals served as controls. The animals were killed under ether anesthesia and blood was collected by heart puncture. A lobe of the liver was rapidly removed for the determination of the activity of antioxidant enzymes and contents of glutathione (GSH) and 4-hydroxyproline. Serum was obtained to determine the levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP). Liver sections fixed in 10% formalin were used for the determination of collagen content.

Preparation and Administration of ZD ZD was isolated from L. chinense (purity >95%). Details of the isolation process were reported elsewhere. ZD was daily given at 10 a.m. per os at a dose of either 12.5 or 25 mg/kg body weight (in 5% Tween 80) throughout the experimental period. Sham-operated rats were given vehicle (5% Tween 80).

Histological Analysis Sections 4 μm thick from the right lobe of the liver were processed routinely for hematoxylin and eosin staining. For the detection of collagen fibers, these sections were further processed for Fast Green/Sirius Red staining.

Determination of Hepatic Collagen Content Collagen content in liver sections was determined by the method of Lopez de Leon and Rojkind, as validated by Jimenez et al.

Determination of 4-Hydroxyproline Content The frozen middle lobes of liver (30—50 mg) were lyophilized overnight. This lyophilized tissue was ground into powder and subsequently extracted five times with 2 ml of diisopropyl ether to remove vitamin A. It was then hydrolyzed with 6 N HCl for 20 h at 100 °C. The hydrolysate was filtered through a 0.22-μm Millipore filter. The content of 4-hydroxyproline in the liver hydrolysate was determined as described.

Fig. 1. Structure of Zeaxanthin Dipalmitate

R : palmitic acid

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Biochemical Analysis  The activities of ALT and AST in
serum were measured according to the method of Reitman
and Frankel using an assay kit obtained locally.15) ALP activity
was measured according to the method of Tietz and Frankel
using an assay kit obtained locally.15) ALT activity was deter-
mined according to the method of Tietz et al.16) Thiobarbituric acid-reactive substances (TBARS) were deter-
mained according to the method of Ohkawa et al.17)

Determination of Antioxidant Enzyme Activity and
Glutathione Level  The activities of glutathione-S-trans-
ferase (GST), superoxide dismutase (SOD), and glutathione
disulfide reductase (GSSGR) in liver tissue were determined
as described elsewhere.18—20) GSH was analyzed fluoromet-
rically by the method of Hissin and Hilf.21) Protein concentra-
tion was determined by the method of Lowry et al. with
bovine serum albumin as the standard.22)

Statistical Analysis  All data are expressed as the
mean±S.D. The evaluation of statistical significance was
determined by the one-way ANOVA test using a standard statistical
package (SAS) for microcomputers.

RESULTS AND DISCUSSION

Hepatic fibrosis is an important feature of chronic liver
disease. Hepatic fibrosis in rats can be induced with BDL for
a duration of one month or longer. The morphological
changes in liver tissue of BDL rats are comparable to those
seen in human biliary fibrosis.23) In the present study, we
attempted to evaluate the extent of the antifibrotic activity of
ZD in a rat model of fibrosis induced by BDL.

BDL in rats for 6 weeks induced a high degree of hepatic
fibrosis. Serum biochemical parameters related to hepatic
function are shown in Table 1. ALT and AST activities in
the serum of sham-operated control rats were 18.7±5.8 and
57.1±11.0 U/l, respectively (mean±S.D.). At week 6, the en-
zyme activities in the serum of BDL rats abruptly increased.
However, the increased serum ALT activity in BDL rats was
significantly decreased when they received ZD at a dose of
25 mg/kg body weight. Serum ALP activity was also
markedly increased in BDL rats. Treatment of BDL rats with
ZD significantly reduced the elevated ALP activity.

At 6 weeks after biliary obstruction, hepatic collagen con-
tent in BDL rats increased to six-fold that in sham-operated
controls (Table 2). Histological examination also showed that
newly formed duct-like structures were associated with cola-
gen deposition after 6 weeks of biliary obstruction. Oral ad-
ministration of ZD 25 mg/kg body weight in BDL rats signif-
ically decreased the collagen content in liver tissue. This
finding was consistent with the reduction in 4-hydroxypro-
line content as assessed by a colorimetric method (Table 2).

The content of TBARS, a marker of lipid peroxidation, in
the serum of BDL rats increased two-fold compared with
that in sham-operated controls. Treatment of BDL rats with
ZD 25 mg/kg body weight significantly reduced the serum
content of TBARS (Table 1). At the cellular level, we have
previously shown that ZD inhibited the proliferation of HSCs
in their MFBLCs and inhibited their collagen synthesis.24) The
addition of conditioned media obtained from primary
cultures of rat hepatocytes injured by CCl4 increased DNA
synthesis up to 2—3-fold in cultured MFBLCs.24) In agree-
ment with those of others,25,26) these results indicate that he-
patocytes undergoing oxidative stress release factors that are
fibrogenic for HSCs. Therefore, taking into account the in
vitro observations, the antifibrotic activity of ZD in vivo may
be due in part to the attenuation of hepatic oxidative stress
and thus inhibit the paracrine effect of damaged hepatocytes.

Several investigators have already shown that the in-
creased concentration of TBARS and a reduction in some an-
tioxidant defenses occur in rats with biliary obstruction, sug-

Table 1. Effects of ZD Treatment on Serum Enzyme Activities and TBARS Levels in BDL Rats

<table>
<thead>
<tr>
<th>Group</th>
<th>AST (U/l)</th>
<th>ALT (U/l)</th>
<th>ALP (U/l)</th>
<th>TBARS (nmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham-operated</td>
<td>57.1±11.0</td>
<td>18.7±5.8</td>
<td>89.6±22.6</td>
<td>3.3±0.3</td>
</tr>
<tr>
<td>BDL</td>
<td>162.0±19.9</td>
<td>71.6±16.5</td>
<td>159.6±44.0</td>
<td>6.4±0.8</td>
</tr>
<tr>
<td>ZD 12.5 mg/kg+BDL</td>
<td>148.8±24.8</td>
<td>70.3±18.3</td>
<td>122.5±17.2</td>
<td>4.3±0.7**</td>
</tr>
<tr>
<td>ZD 25.0 mg/kg+BDL</td>
<td>143.6±18.3*</td>
<td>65.7±21.4</td>
<td>80.2±36.2***</td>
<td>3.8±1.5**</td>
</tr>
</tbody>
</table>

BDL represents rats with biliary obstruction. ZD was given per os at a dose of either 12.5 or 25.0 mg/kg/d during weeks 1—6 (both n=8). Sham-operated rats were given vehicle (5% Tween 80, n=5). Data are expressed as mean±S.D. a) Significantly different from sham-operated controls (p<0.005). *,**,*** Significantly different from BDL controls at p<0.05, p<0.01, and p<0.001, respectively.

Table 2. Effects of ZD Treatment on Contents of Hydroxyproline and Col-
lagen in BDL Rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Hydroxyproline content (mg/g total liver)</th>
<th>Collagen content (µg/mg total protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham-operated</td>
<td>2.5±0.8</td>
<td>25.1±4.3</td>
</tr>
<tr>
<td>BDL</td>
<td>31.7±6.5*</td>
<td>155.5±12.9*</td>
</tr>
<tr>
<td>ZD 12.5 mg/kg+BDL</td>
<td>25.6±3.8*</td>
<td>153.2±13.1</td>
</tr>
<tr>
<td>ZD 25.0 mg/kg+BDL</td>
<td>20.7±2.7**</td>
<td>134.4±9.0**</td>
</tr>
</tbody>
</table>

BDL was given per os at a dose of either 12.5 or 25.0 mg/kg/d during weeks 1—6 (both n=8). Sham-operated rats were given vehicle (5% Tween 80, n=5). Collagen content in liver sections was measured by the selective binding of Sirius red and Fast Green to collagen and non-collagen protein, respectively. Total 4-hydroxyproline con-
tent in liver hydrolysates was determined spectrophotometrically as described in Mate-
rials and Methods. Data are expressed as mean±S.D. a) Significantly different from sham-operated controls (p<0.005). *,**,*** Significantly different from BDL controls at p<0.05 and p<0.01, respectively.
fect of ZD in BDL rats, although their conventional laboratory test results changed only slightly.

The results presented here showed that BDL rats are profoundly deficient in antioxidant enzymes in addition to GSH deficiency (Table 3). These findings also suggest that the diseased liver may have a diminished capacity to scavenge free radicals and that lipid peroxidation may be associated with cholestatic liver injury. Biliary obstruction decreased the activity of SOD in liver tissue to 35% of the sham-operated control level and treatment of BDL rats with ZD slightly reduced this enzyme activity, but the reduction was not statistically significant. There was also no significant difference in the activity of GSSGR between sham-operated controls, BDL rats, and BDL rats treated with ZD. However, GST activity in BDL rats treated with ZD was significantly increased compared with that in BDL rats (Table 3). GST can bind bilirubin, the toxic breakdown product of hemoglobin, or cytochrome P450s, which are elevated in cholestatic liver injury. Biliary obstruction decreased the activity of GSSGR between sham-operated controls, ZD 25.0 mg/kg + BDL. However, GST activity in BDL rats treated with ZD slightly reduced the toxicity induced by bilirubin.32) Therefore the preservation of GST activity in BDL rats upon the treatment with ZD may mitigate the toxicity induced by bilirubin.

In conclusion, the antifibrotic activity of ZD appears to be mediated via inhibition of the proliferation of MFBLCs, by inhibition of the paracrine signaling transmitted from damaged hepatocytes and activated Kupffer cells, by reducing the oxidative stress induced by BDL, and partially by decreased bilirubin toxicity due to the preservation of GST activity. Therefore ZD may be a major antifibrotic constituent of *L. chinense*. Recent observations suggested that BDL resulted in the inactivation of metalloproteinases, such as collagenase, and the induction of tissue inhibitors of metalloproteinases.33) To determine the precise roles played by ZD in the suppression of fibrogenesis, its effect on the degradation of extracellular matrix proteins should be investigated further.

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**REFERENCES AND NOTES**

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<table>
<thead>
<tr>
<th>Group</th>
<th>GSH (nmol/mg protein)</th>
<th>GST (nmol/mg protein/min)</th>
<th>GSSGR (μmol/mg protein/min)</th>
<th>SOD (U/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham-operated</td>
<td>56.9 ± 10.5</td>
<td>28.9 ± 2.5</td>
<td>11.6 ± 2.4</td>
<td>4.6 ± 0.5</td>
</tr>
<tr>
<td>BDL</td>
<td>10.2 ± 5.9*</td>
<td>19.7 ± 4.6*</td>
<td>11.1 ± 3.8</td>
<td>3.0 ± 0.4*</td>
</tr>
<tr>
<td>ZD 12.5 mg/kg + BDL</td>
<td>26.8 ± 16.4*</td>
<td>29.6 ± 5.1*</td>
<td>12.1 ± 5.7</td>
<td>3.2 ± 0.3</td>
</tr>
<tr>
<td>ZD 25.0 mg/kg + BDL</td>
<td>44.5 ± 11.9***</td>
<td>32.0 ± 4.3*</td>
<td>12.8 ± 3.1</td>
<td>3.4 ± 0.2</td>
</tr>
</tbody>
</table>

ZD was given per os at a dose of either 12.5 or 25 mg/kg/d during weeks 1–6 (n = 8). Sham-operated rats were given vehicle (5% Tween 80, n = 5). Data are expressed as mean ± S.D. *a) Significantly different from sham-operated controls (p < 0.05). b) Significantly different from sham-operated controls (p < 0.005). *c) Significantly different from BDL controls at p < 0.05 and p < 0.001, respectively.

Table 3. Effects of ZD Treatment on Hepatic Antioxidant Enzymes in BDL Rats