Differential Mechanisms of Increased $\alpha_1$-Fetoprotein Production in Rats following Carbon Tetrachloride Injury and Partial Hepatectomy

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SUMMARY

Possible differences in the mechanisms of increased $\alpha_1$-fetoprotein (AFP) production following carbon tetrachloride (CCl$_4$) intoxication and partial hepatectomy were studied with 5-week-old rats at the time of sacrifice. The maximum level of serum AFP reached in 4 days after a single dose of CCl$_4$ was much higher than that after partial hepatectomy, although the incorporation of $[^3H]$thymidine into liver DNA increased nearly to the same extent by either of these treatments. In the remnant after partial hepatectomy, the DNA synthesis that was further accelerated by treatment with a lower dose of thioacetamide was not associated with any further increase of serum AFP levels. However, CCl$_4$ given to partially hepatectomized rats had an additive effect on increased AFP levels. The increases of serum AFP concentrations in CCl$_4$-injured rats were depressed by Mitomycin C given in vivo, whereas the increases in partially hepatectomized rats were not. Treatment with 8-azaguanine inhibited both increases of serum AFP levels, although the inhibition was much less or was insignificant in partially hepatectomized rats. These results suggest the existence of different underlying mechanisms of the increased AFP production for the two experimental conditions.

INTRODUCTION

Raised AFP concentrations in serum during the course of hepatitis and cirrhosis of the liver have been suggested to be caused by liver regeneration (6, 13). This consideration is based on the comparison between temporal alterations in AFP level and serum GPT activity (3, 7, 8) and also on the fact that, in rats and mice, the serum AFP level increased coincidentally at the time of liver regeneration after partial hepatectomy and CCl$_4$ intoxication (1-3, 11). However, few direct comparisons of the mechanisms of AFP production in rats following partial hepatectomy and CCl$_4$ injury have been reported, probably because the increased AFP level has been considered to be due simply to the hepatocyte regeneration following CCl$_4$-induced necrosis of liver cells. The increased production of AFP in rats treated with CCl$_4$ and other hepatotoxins has been shown to be closely associated with liver cell injury per se and not with the stimulated synthesis of DNA in damaged liver, as described in our recent paper (19). Furthermore, our previous studies of key hepatic glycolytic and gluconeogenic enzymes in rats treated with CCl$_4$ and partial hepatectomy have revealed a much greater extent of enzyme deviations in the injured liver than in the liver after partial hepatectomy, this being again caused by hepatic cell injury and not by liver regeneration (16). These results could be interpreted as providing a biochemical basis for the repair process of liver injury, which is distinct from the liver regeneration after partial hepatectomy.

In the present work, comparative studies were made on the serum AFP level and liver DNA synthesis in rats following CCl$_4$ injury and partial hepatectomy. Different effects of Mitomycin C and 8-azaguanine on the increased production of AFP under these experimental conditions are also described in this report.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats were obtained from Awazu Experimental Animal Co., Osaka, Japan, and were kept on Oriental Laboratory Chow MF and water ad libitum. Rats were fasted overnight and then given CCl$_4$ (Katayama Chemical Co., Tokyo, Japan) by intubation at a dose of 0.5 ml of 20% solution in liquid paraffin per 100 g body weight, unless otherwise stated. Control rats received an equivalent volume of liquid paraffin alone. After the administration, treated and control animals were fasted for 2 days and then fed on laboratory chow and water ad libitum until sacrificed at the time indicated. Partial hepatectomy was performed under ether anesthesia according to the method of Higgins and Anderson (4). Hepatectomized rats were maintained on the basal diet given ad libitum until sacrificed. For the combined treatment of partial hepatectomy and poisoning, 1 ml of 20% CCl$_4$ in liquid paraffin or 0.4% thioacetamide (E. Merck AG, Darmstadt, Germany) in 0.9% NaCl solution was given to rats i.g. and i.p. immediately after or 1 hr after partial hepatectomy, respectively. Experiments were designed so that all the treated rats were sacrificed at 35 days...
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of age; considerably high levels of serum AFP were obtained under these conditions, which are beneficial for investigating the detailed mechanisms of increased AFP production (19). Mitomycin C (Kyowa Hakko Kogyo, Co., Tokyo) was administered i.p. in 2 equal doses of 0.22 mg/100 g body weight; the 1st dose was given at 6 hr and the 2nd dose at 48 hr after CCl₄ administration or partial hepatectomy. 8-Azaguanine ([lSigma Chemical Co., St. Louis, Mo.] dissolved in 1 N NaOH at a concentration of 8 mg/ml, pH adjusted to 8.5 with dilute HCl] was injected i.p. at 1, 2, and 3 days after CCl₄ treatment or partial hepatectomy, each time in a dose of 4 mg per 100 g body weight.

Analytical Procedures. The serum AFP concentrations were determined by a double-antibody technique with [¹³¹I]- or [¹²⁵I]-labeled AFP, which was purified from rat fetal serum (19). The incorporation of [methyl-³H]thymidine (The Radiochemical Centre, Amersham, England [specific activity, 18.4 Ci/mmmole]) into hepatic DNA was estimated according to the method of Schmidt and Thanhauser (14) by an i.p. injection of 70 μCi of [³H]thymidine per 100 g body weight 1 hr before sacrifice. The specific radioactivity of labeled DNA was measured as described in detail in our previous paper (19). Serum GPT and liver G6PD activities, liver triglyceride, and protein contents were determined as previously described (19) to estimate the extent of liver damage. All the results were expressed as mean ± S.E. Significant differences between the mean values were determined by Student's t test after analysis of variance.

RESULTS

Time Courses of Increases in Serum AFP Concentration and Incorporation of [³H]Thymidine into Liver DNA following CCl₄ Treatment and Partial Hepatectomy. Time courses of increases in the serum AFP concentration and hepatic DNA synthesis following acute CCl₄ intoxication and partial hepatectomy were compared directly (Charts 1 and 2). In CCl₄-injured rats, the serum AFP concentration increased significantly in 2 days, reached a maximum mean value in approximately 4 days, and then started to decrease gradually. AFP levels were still elevated on Day 7. On the other hand, the increased incorporation of [³H]thymidine into DNA reached a maximum level in 3 days and fell rapidly to the normal level within 7 days.

In contrast to this, the increase in serum AFP concentration after partial hepatectomy was very slight; it reached a nearly maximum value in 4 days and returned to the initial level on Day 7 (Chart 2). The extent of total incorporation of [³H]thymidine into hepatic DNA following partial hepatectomy was even greater than that after CCl₄ treatment, but a peak height of hepatic DNA synthesis was found to be nearly the same under both experimental conditions. A greater than 10-fold increase in DNA synthesis was observed as early as 24 hr after partial hepatectomy, whereas no significant increase was detected by 24 hr of CCl₄ intoxication. Control rats treated with liquid paraffin alone and sham-operated animals gave no appreciable changes in the AFP level under identical experimental conditions.

Effect of CCl₄ or Thioacetamide Treatment on Serum AFP Concentration and Incorporation of [³H]Thymidine into Liver DNA in Partially Hepatectomized Rats. The combined effects of partial hepatectomy and CCl₄ intoxication on the serum AFP concentration and liver DNA synthesis were studied by giving twice the amount of CCl₄ immediately after partial removal of the liver (17) (cf. 'Materials and Methods'). The partially hepatectomized rats seemed to be equally sensitive to this dose of CCl₄, compared with the untreated rats receiving the standard dose of CCl₄ on the basis of similar alterations of liver function tests in 24 hr (Table 1). Only serum GPT activities of dually treated rats were less than those of CCl₄-injured rats without hepatectomy (p < 0.005). The liver weights of hepatectomized rats were not markedly different from those of dually treated rats, both 2 and 4 days after the operation. However, serum AFP levels in hepatectomy plus CCl₄ treatment were much higher than those in hepatectomy alone (p < 0.005) and were close to those in CCl₄ treatment alone (Table 2). When mean values were compared, the combined effect of CCl₄ treatment and partial hepatectomy could be interpreted as additive, although the extent of increase in hepatic DNA synthesis was even slightly less in the dually treated rats than in hepatectomized or CCl₄-treated rats.

The administration of a lower dose of thioacetamide reportedly stimulates DNA synthesis and cell proliferation without any evidence of liver injury (12, 19). The increased synthesis of liver DNA in hepatectomized rats was further stimulated by the simultaneous administration of a lower dose of thioacetamide (4 mg/100 g body weight) (p < 0.05), as was observed by Mironescu and Ciovirnache (10). However, serum AFP concentrations in hepatectomized and
then thioacetamide-treated rats failed to show any further increases.

**Effect of Mitomycin C or 8-Azaguanine on Serum AFP Level following CCl₄ Intoxication and Partial Hepatectomy.**

To determine the effect of inhibiting DNA synthesis on serum AFP levels, Mitomycin C was administered twice, 6 and 24 hr after CCl₄ treatment or partial hepatectomy (5). Under the conditions of Mitomycin C administration given in "Materials and Methods," the inhibition of DNA synthesis ($p < 0.005$) was about 80% in both CCl₄ injury and partial hepatectomy (Table 3). The liver weights of Mitomycin C-treated rats were much smaller than those in Mitomycin C-un-treated animals. The experimental observations that Mitomycin C did not influence the pathogenesis of CCl₄-induced liver injury have been published in our previous paper (18). Mitomycin C significantly inhibited the increases in serum AFP concentration after CCl₄ intoxication ($p < 0.05$), whereas it failed to inhibit the increases after partial hepae-

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**Table 1**

Effect of CCl₄ on biochemical parameters for liver injury in partially hepatectomized rats

Animals were sacrificed 2 days after partial hepatectomy and CCl₄ intoxication. There were 5 rats in each treatment group.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Liver wt. (g)</th>
<th>Serum GPT (K.U.)</th>
<th>Liver triglyceride (mg/g liver)</th>
<th>Liver protein (mg/g liver)</th>
<th>Liver G6PD (milli-units/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>3.4 ± 0.3b</td>
<td>30 ± 9</td>
<td>3 ± 1</td>
<td>199 ± 5</td>
<td>32 ± 1</td>
</tr>
<tr>
<td>Hepatectomy</td>
<td>2.8 ± 0.2</td>
<td>48 ± 5</td>
<td>7 ± 2</td>
<td>167 ± 5</td>
<td>55 ± 8a</td>
</tr>
<tr>
<td>Hepatectomy + CCl₄</td>
<td>2.4 ± 0.1c</td>
<td>263 ± 26d</td>
<td>38 ± 6c</td>
<td>156 ± 12c</td>
<td>101 ± 5f</td>
</tr>
<tr>
<td>CCl₄</td>
<td>4.6 ± 0.1c</td>
<td>1012 ± 137e</td>
<td>45 ± 4f</td>
<td>152 ± 4f</td>
<td>110 ± 3f</td>
</tr>
</tbody>
</table>

* K.U., Karmen units.
* Mean ± S.E.
* $p < 0.05$.

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**Table 2**

Effect of CCl₄ and thioacetamide on serum AFP level and incorporation of [³H]thymidine into liver DNA in partially hepatectomized rats

There were 5 rats in each treatment group. The incorporation of [³H]thymidine into liver DNA was determined 3 days after treatments. Other results were obtained after 4 days of the treatments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Liver wt. (g)</th>
<th>Serum GPT (K.U.)</th>
<th>Serum AFP (ng/ml)</th>
<th>Specific radioactivity of DNA (cpm/μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>3.0 ± 0.1a</td>
<td>28 ± 6</td>
<td>160 ± 39</td>
<td>13 ± 1 (2)a</td>
</tr>
<tr>
<td>Hepatectomy</td>
<td>3.1 ± 0.1</td>
<td>32 ± 4</td>
<td>810 ± 353d</td>
<td>178 ± 11c (3)</td>
</tr>
<tr>
<td>Hepatectomy + CCl₄</td>
<td>3.4 ± 0.1</td>
<td>55 ± 5'</td>
<td>6721 ± 1785f</td>
<td>153 ± 3' (2)</td>
</tr>
<tr>
<td>CCl₄</td>
<td>4.3 ± 0.1c</td>
<td>135 ± 42c</td>
<td>5878 ± 1844c</td>
<td>215 ± 10d (3)</td>
</tr>
<tr>
<td>Hepatectomy + thioacetamide</td>
<td>3.6 ± 0.3</td>
<td>32 ± 8</td>
<td>678 ± 208c</td>
<td>303 ± 47' (2)</td>
</tr>
<tr>
<td>Thioacetamide</td>
<td>4.1 ± 0.2c</td>
<td>40 ± 7</td>
<td>230 ± 102</td>
<td>96 ± 26' (3)</td>
</tr>
</tbody>
</table>

* K.U., Karmen units.
* Mean ± S.E.
* $p < 0.05$.
* Numbers in parentheses, number of rats used exclusively for determination of specific radioactivity of DNA.

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**Table 3**

Effect of Mitomycin C on serum AFP level and incorporation of [³H]thymidine into liver DNA in CCl₄-injured and partially hepatectomized rats

Relative liver weights and serum AFP concentrations were obtained 4 days after partial hepatectomy or CCl₄ intoxication. The incorporation of [³H]thymidine into liver DNA was studied 3 days after the treatments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of rats</th>
<th>Liver wt. (g)</th>
<th>Serum AFP (ng/ml)</th>
<th>Specific radioactivity of liver DNA (cpm/μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>4</td>
<td>3.3 ± 0.1a</td>
<td>160 ± 39</td>
<td>13 ± 1 (2)</td>
</tr>
<tr>
<td>Mitomycin C</td>
<td>5</td>
<td>2.3 ± 0.2</td>
<td>105 ± 16</td>
<td>5 ± 1 (2)</td>
</tr>
<tr>
<td>Hepatectomy</td>
<td>4</td>
<td>3.0 ± 0.3</td>
<td>1049 ± 239b</td>
<td>178 ± 11h (3)</td>
</tr>
<tr>
<td>Hepatectomy + Mitomy-</td>
<td>3</td>
<td>1.5 ± 0.1a</td>
<td>2593 ± 1118f</td>
<td>39 ± 5h (3)</td>
</tr>
<tr>
<td>CCl₄</td>
<td>5</td>
<td>5.4 ± 0.3c</td>
<td>4060 ± 111b</td>
<td>215 ± 10h (3)</td>
</tr>
<tr>
<td>CCl₄ + Mitomycin C</td>
<td>3</td>
<td>4.2 ± 0.1</td>
<td>1880 ± 336c</td>
<td>49 ± 4h (2)</td>
</tr>
</tbody>
</table>

* Mean ± S.E.
* $p < 0.05$.
* Numbers in parentheses, numbers of rats used exclusively for determination of specific radioactivity of DNA.
Effects of a guanine antagonist, 8-azaguanine, on serum AFP levels in CCl₄-injured or partially hepatectomized rats were summarized in Table 4. The dose of 8-azaguanine used in this experiment has been reported to be sufficient to inhibit the dietary induction of hepatic G6PD and malic enzyme (15). However, the increase in G6PD activity in CCl₄-injured rat liver was not inhibited by the simultaneous administration of 8-azaguanine, in addition to its failure to alter the elevations of serum GPT activity and liver triglyceride content induced by CCI₄, suggesting that the pathogenesis of CCI₄-induced liver damage is not interfered with by this drug. Treatments with 8-azaguanine significantly inhibited the increase in serum AFP concentration following a single dose of CCI₄ (p < 0.025). The inhibitory effect was much less in the partially hepatectomized rats, the difference in the mean value being statistically insignificant.

**DISCUSSION**

In assessment of the role of hepatic regeneration in increased AFP production in CCI₄ liver injury, it is practically impossible to separate the stage or phase of degenerative and regenerative changes of liver cells. In order to circumvent this difficulty, a direct comparison of the increased AFP production and accelerated hepatic DNA synthesis was made in CCl₄-injured rats and partially hepatectomized rats. Acute CCl₄ intoxication of rats caused a marked elevation of serum AFP, as compared with partial hepatectomy. In order to explain the large difference, a much greater increase in hepatic DNA synthesis would be expected for CCl₄-injured liver, assuming that the increased number of liver cell after regeneration is responsible for the increased AFP production. However, no marked difference in [³H]thymidine incorporation into hepatic DNA between the 2 experimental conditions was found in this study. Slight increases in the serum AFP concentration in hepatectomized rats, compared with those in CCl₄-treated rats, may be due to the smaller amount of liver left after the operation. However, this possibility was eliminated by the finding that the combined effects of partial hepatectomy and CCl₄ treatment on serum AFP levels were additive. The greater increase in AFP concentration in dually treated rats was not accompanied by further increase of DNA synthesis over either of the values obtained by single treatments. Until 7 days, the serum AFP concentrations remained higher in CCl₄-treated rats than in partially hepatectomized rats, although the increased liver DNA synthesis fell sharply to normal in both treatments. The persistence of higher levels of serum AFP in the late stage of CCl₄ intoxication cannot be adequately explained by assuming a direct relationship between AFP production and stimulated DNA synthesis. Furthermore, the marked acceleration of DNA synthesis by treating hepatectomized rats with a lower dose of thioacetamide failed to give a further increase of serum AFP level. Similarly, a lower dose of thioacetamide given to untreated rats caused enhanced hepatic DNA synthesis without inducing any cellular damage of liver and, again, serum AFP level remained low (19). A single injection of ethionine, which caused elevated levels of liver triglyceride and G6PD to occur without liver cell necrosis, produced a remarkable elevation of serum AFP level prior to the stimulation of hepatic DNA synthesis (19). As a result of these observations, it may be suggested that some additional mechanism, which is in close connection with liver cell injury and which leads to an increased AFP production per cell, plays a major role in the increased production of AFP in CCl₄-injured liver. Thus, the stimulated synthesis of DNA in injured liver may not be directly related to the elevation of serum AFP level.

Since the elevation of serum AFP concentrations following CCl₄ administration was inhibited by either Mitomycin C or 8-azaguanine, RNA synthesis de novo appears to be a prerequisite for the CCl₄-induced increase in AFP production. Mitomycin C has been reported to inhibit the syntheses of both DNA and RNA to the same extent (9). Accordingly, an AFP-specific gene amplification would be brought about by the liver injury at the level of transcription, resulting in an increased AFP synthesis. A concomitant stimulation of DNA synthesis would ensue as a part of repair process of injured liver cells. The relatively small increase in serum AFP level after partial hepatectomy would represent an AFP synthesis by utilization of preexisting mRNA in some way geared with

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**Table 4**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of rats</th>
<th>Serum GPT (K.U.)*</th>
<th>Liver G6PD (munits/mg protein)</th>
<th>Serum AFP (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>2</td>
<td>32 ± 3*</td>
<td>36 ± 3</td>
<td>120 ± 41</td>
</tr>
<tr>
<td>8-Azaguanine</td>
<td>2</td>
<td>31 ± 1</td>
<td>34 ± 13</td>
<td>173 ± 29</td>
</tr>
<tr>
<td>Hepatectomy</td>
<td>5</td>
<td>32 ± 4</td>
<td>67 ± 8</td>
<td>810 ± 353*</td>
</tr>
<tr>
<td>Hepatectomy + 8-azaguanine</td>
<td>6</td>
<td>33 ± 4</td>
<td>76 ± 4*</td>
<td>589 ± 192*</td>
</tr>
<tr>
<td>CCl₄</td>
<td>6</td>
<td>576 ± 340</td>
<td>71 ± 13*</td>
<td>5242 ± 340*</td>
</tr>
<tr>
<td>CCl₄ + 8-azaguanine</td>
<td>5</td>
<td>380 ± 42*</td>
<td>82 ± 15*</td>
<td>1423 ± 537*</td>
</tr>
</tbody>
</table>

*K.U., Karmen units.

*M Mean ± S.E.

*p < 0.05.
a new cycle of cell proliferation, thus making it clearly distinct from the marked increase in AFP synthesis after CCl₄ treatment, which involves a gene activation at the level of transcription.

REFERENCES

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