Hepatic Regeneration after Sublethal Partial Liver Irradiation in Cirrhotic Rats

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Hepatic regeneration/Liver cirrhosis/Irradiation.

Our previous animal study had demonstrated that partial liver irradiation (IR) could stimulate regeneration in the protected liver, which supported the measurements adopted in radiotherapy planning for hepatocellular carcinoma. The purpose of this present study is to investigate whether cirrhotic liver repopulation could be triggered by partial liver IR. The cirrhosis was induced by thioacetamide (TAA) in rats. After cirrhosis establishment, TAA was withdrawn. In Experiment 1, only right-half liver was irradiated with single doses of 5 Gy, 10 Gy and 15 Gy, respectively. In Experiment 2, right-half liver was irradiated to 15 Gy, and the left-half to 2.5 Gy, 5 Gy and 7.5 Gy, respectively. The regeneration endpoints, including liver index (LI); mitotic index (MI); liver proliferation index (LPI); PCNA-labeling index (PCNA-LI); serum HGF, VEGF, TGF-α and IL-6, were evaluated on 0 day, 30-day, 60-day, 90-day, 120-day and 150-day after IR. Serum and in situ TGF-β1 were also measured. In both experimental groups, the IR injuries were sublethal, inducing no more than 9% animal deaths. Upon TAA withdrawal, hepatic regeneration decelerated in the controls. In Experiment 1 except for LI, all other regeneration parameters were significantly higher than those in controls for both right-half and left-half livers. In Experiment 2 all regeneration parameters were also higher compared with those in controls for both half livers. Serum HGF and VEGF were increased compared with that of controls. Both unirradiated and low dose-irradiated cirrhotic liver were able to regenerate triggered by sublethal partial liver IR and higher doses and IR to both halves liver triggered a more enhanced regeneration.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world.1,2 As over 70% of Chinese HCC are not candidates for surgery, radiation therapy (RT) becomes an option for their treatments. The RT efficacy by three-dimensional conformal radiotherapy (3DCRT) and intensity modulated radiation therapy (IMRT) for locally advanced HCC has been encouraging with a 3-year overall survival of 26%–35%.3–5 However, radiation-induced liver disease (RILD), the most severe and almost fatal complica-

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MATERIALS AND METHODS

Animals
The study was approved, and the care and use of animals were in accordance with Fudan University Guidelines and Regulations on the Use and Care of Lab Animals. Pathogen-free male Wistar rats obtained from the Division of Laboratory Animal Medicine, Fudan University, weighing 200 ± 15 g, were used. The animals were acclimatized to our laboratory conditions at temperature, 22°C and humidity, 55%. The rats were given free access to diets and water ad libitum.

Induction of liver cirrhosis
Rats were given thioacetamide (TAA) (Aladdin-Reagent Shanghai Ltd., China) in their drinking water at a concentration of 0.03% throughout the establishment of cirrhosis. Three rats were sacrificed each time at a three-week interval to monitor the development of cirrhosis from the 21st week onwards after TAA feeding until all 3 rats showed overt cirrhosis. The cirrhosis was confirmed by histological feature, trichrome stain and quantification of hydroxyproline in hydrolysed liver tissue. Upon the successful establishment of cirrhosis TAA water was withdrawn and replaced by natural drinking water. Then the cirrhotic rats were ready for experiments. Thirty rats, drinking natural water, were randomly chosen as healthy control.

Experiment design
The study consisted of two experiments (Table 1). All control groups (without IR) were designated 0–0 Gy.

<table>
<thead>
<tr>
<th>Experiment and subgroup</th>
<th>Dose to Right-half liver</th>
<th>Dose to Left-half liver</th>
<th>Mortalities</th>
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<tr>
<td>Healthy rat (30 rats)</td>
<td>0 Gy</td>
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<td>Control (25 rats)</td>
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<td>5–0 Gy (25 rats)</td>
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<td>10–0 Gy (30 rats)</td>
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<tr>
<td>15–0 Gy (30 rats)</td>
<td>15 Gy</td>
<td>0 Gy</td>
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<tr>
<td>Experiment 1 (110 rats with cirrhosis)</td>
<td>15–0 Gy (30 rats)</td>
<td>15 Gy</td>
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<tr>
<td>15–2.5 Gy (30 rats)</td>
<td>15 Gy</td>
<td>2.5 Gy</td>
<td>0</td>
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<tr>
<td>15–5 Gy (34 rats)</td>
<td>15 Gy</td>
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<td>5.9% (2/34)</td>
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<tr>
<td>15–7.5 Gy (36 rats)</td>
<td>15 Gy</td>
<td>7.5 Gy</td>
<td>8.3% (3/36)</td>
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IR
Rats were anesthetized with intraperitoneal injection (i.p.) of 2% pentobarbital (35 mg/kg) and irradiated in prone position with 6 MV-X ray at a dose rate of 3.15 Gy/min. IR field was marked via simulator fluoroscope with superior margin on diaphragm dome, inferior margin at costal arch on each side, and the lateral margin covering both abdominal edges, while the rest of rat was protected by lead shield. The vertebral line was taken as the separating line for the left- and right-half liver. IR dose was calculated at liver middle plane upon the measurements by ion chamber in a phantom.

Sample collection
Blood samples and liver tissues were collected immediately after animals were sacrificed under 2% pentobarbital solution (50 mg/kg, i.p.) anesthesia according to schedule. 8–10 ml whole blood was collected from the heart chamber, and the serum was collected after centrifugation at 3,000 rpm for 10 min. Two pieces of liver tissues, 0.5 cm × 0.5 cm × 0.5 cm each, were collected from both right- and left-half livers.

Endpoints to evaluate hepatic injury and regeneration
The following endpoints were evaluated: (1). Liver injury: Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP). (2). Cirrhosis: Quantitative hydroxyproline in hydrolysed liver tissue, collagen in liver tissue, and serum and in situ transforming growth factor beta-1 (TGF-β1). (3). Liver regeneration: Liver index (LI, liver and total body weights ratio); mitotic index (MI); liver proliferation index (LPI, the percentage of cells at S and G2/M phases in total number of cells); proliferating cell nuclear antigen (PCNA)-labeling index (PCNA-LI). (4). Serum growth factors: Hepatic growth factor (HGF), vascular endothelial growth factor (VEGF), transforming growth factor-alfa (TGF-α) and interleukin-6 (IL-6). Also, the animal deaths induced by treatments were observed.

Biochemistry assay
(1). Serum biochemistry was measured by HITACHI biochemical analyzer and determined using standard kits for ALT, AST and ALP (Shino-test Corporation, Tokyo, Japan); (2). Serum concentrations of HGF, TGF-β1, VEGF, TGF-α and IL-6 were measured by ELISA kits (RapidBio Lab, CA, USA); (3). Quantitative hydroxyproline in hydrolysed liver
RESULTS

TAA-induced cirrhosis

TAA-induced cirrhosis was successfully established at week 29 after drinking TAA water, confirmed both macro and microscopically, of which the collagen and hydroxyproline contents were significantly higher compared with the healthy liver (31.8 ± 6.2% and 4209.6 ± 513.2 μg/g respectively, vs. 1.3 ± 0.4% and 272.5 ± 72.7 μg/g, respectively, \( P < 0.01 \)) (Fig. 1a and b).

Mortalities and liver function impairment

During the whole experimental period, there were minor rat deaths. The mortality rates were no more than 9% (Table 1).

For the controls (0–0 Gy), liver dysfunction improved upon TAA withdrawal with decrease of ALT, AST and ALP but did not resume to the levels as that of the healthy rats on d120 (Fig. 2a).

For rats with right-half liver IR (Experiment 1), recovery of liver dysfunction was noted to decelerate after TAA withdrawal as compared with the controls, and the higher the irradiation dose, the slower recovery to that of the control level as noted on d120 (Fig. 2a). For rats with both right- and left-half liver irradiation (Experiment 2), recovery of liver dysfunction was even slower, and higher IR dose resulted in persistent worsening of liver function after TAA withdrawal. Lower ALT and AST levels on d150 compared with d0 were noted but still higher than the controls on d150. ALP level was noted to be similar to the control on d150 (Fig. 2b).

Hepatic regeneration

Experiment 1

LPl, PCNA-LI, MI and LI of the control group on d0 were significantly higher than that in healthy rats \((P < 0.05)\), confirming TAA as a stimulant for liver regeneration (Fig. 3 and 4). The observed dynamic changes were similar between both the left- and right-half livers and thus the overall trends and patterns were described.

In IR groups, LPls decreased slightly and increased from d90 to d120, which was significantly higher than that of the control \((P < 0.05)\) but no differences was noted among other groups. PCNA-LI decreased significantly on d30 \((P < 0.05)\) and increased again but did not reach the similar level on d0. PCNA-LIs were always high in the irradiated rats compared with that of the control \((P < 0.05)\) except for the 5–0 Gy group on d30 and d60 in the left-half liver. MIs decreased after d0, but significantly higher than the control, most notable on d60, d90 and d120 \((P < 0.05)\). Similar trends that higher IR dose yielded higher value were observed for both PCNA-LI and MI.

LIs of the irradiated rats decreased and remained at similar levels to that of the control, except for the 15–0 Gy group
Fig. 1. Features of normal and TAA-induced cirrhosis. (a) Numerous irregular nodules was noted on the surface of the TAA-induced cirrhotic liver, with fully developed cirrhotic nodules surrounded by thick fibrous septa in most part of the liver. Evident collagen fiber (Trichrome stain, 100x), remarkable TGF-β1 and PCNA expression (IHC stain, 200x) in the cirrhotic liver by IHC stain. (b) Quantification of collagen and hydroxyproline contents in normal and cirrhotic liver tissue on d0. Data are expressed as means ± SD. *P < 0.01.

Fig. 2. Changes of liver functions after TAA withdrawal. (a) Experiment 1. (B) Experiment 2.
Experiment 1

On d60, which was remarkably higher than that of others ($P < 0.05$) (Fig. 4a).

Experiment 2

The observed dynamic changes of all regeneration endpoints were similar between both the left- and right-half livers and thus only the overall trends and patterns were described (Fig. 5).

LPIs decreased slightly and increased to peak on d120 for 15–2.5 Gy, 15–5 Gy and 15–7.5 Gy groups compared with those of the control ($P < 0.05$). Decrease in PCNA-LIs was noted for all IR groups after d0. MIs decreased slowly after d0 and no differences noted among all IR groups but were always higher than that of the control at all time points, notably on d30 and d60 ($P < 0.05$).
LIs of the irradiated rats remained slightly decreased throughout the observation period but were always significantly higher than the controls ($P < 0.05$) from d60 till d150 (Fig. 4b).

**HGF, IL-6, VEGF and TGF-α in serum**

Changes in serum growth factors were similar for Experiment 1 and 2 except for IL-6.

**HGF, VEGF and TGF-α**

Serum HGF of the control, higher at baseline, increased slightly and then decreased to the levels similar to the healthy rats from d60 onwards (Fig. 6a). No difference was noted between the control and the healthy rats at all observed time points for serum VEGF and TGF-α (Fig. 6a).

Notable increase of the HGF concentration of the right-half liver IR groups on d60 and d90, and that of the right- and left-half liver IR groups from d60 to d150 was observed (Fig. 6). VEGF also increased greatly on d60 except for 5-0 Gy group. No changes were noted for TGF-α concentrations throughout the observation period.

**IL-6**

No change was noted for serum IL-6 in Experiment 1 but significant increase after IR was observed for 15-0 Gy group in Experiment 2 (Fig. 6).

**TGF-β1 in serum and in situ**

Comparison between the controls and the healthy rats revealed higher serum TGF-β1 and stronger in situ TGF-β1 expression among the controls on d0 with gradual decrease of serum level upon TAA withdrawal but remained significantly higher while TGF-β1 IHC index declined to level similar to that of the healthy rats (Fig. 1a, 6 and 7a).

**Experiment 1**

Serum level of TGF-β1 for all IR groups, higher than that of the control, remained the same throughout the observation period. TGF-β1 IHC index expression was higher than that of the control in both half livers but trend of higher IR doses resulted in more higher values in the right-half (irradiated) was observed (Fig. 6 and 7a).

**Experiment 2**

Results were almost similar to that of Experiment 1 for serum TGF-β1 and TGF-β1 IHC index in the IR groups but the extent of increase were more remarkable and higher IR doses resulted in stronger TGF-β1 expression (Fig. 6 and 7b).

**DISCUSSION**

Radiotherapy for the management of HCC in patients with cirrhosis has been a challenge. Delayed liver regeneration and substantially lesser regenerated volume in cirrhotic patients after partial hepatectomy had been observed, and compensatory hypertrophy of the radiation-spared section...
Fig. 6. Changes of serum growth factor. (a) Experiment 1; (b) Experiment 2. HGF concentration increased notably on d60 and d90 (Experiment 1) \((P < 0.05)\), and from d60 to d150 (Experiment 2) compared with those of 0–0 Gy group \((P < 0.05)\). VEGF increased greatly on d60 \((P < 0.05)\) except for the 5–0 Gy group. TGF-\(\alpha\) concentration did not change significantly during the observation period. Serum IL-6 did not change greatly in Experiment 1 but significant increase was noted after IR in Experiment 2 except for the 15–0 Gy group.
after proton radiation was reported in cirrhotic HCC patients.10,11 Thus, we carried out this study to investigate the regeneration capacity of cirrhotic liver after partial liver IR and its kinetics.

Key to the first step was to establish a cirrhotic animal model. Prolonged exposure to TAA has been reported to result in liver cirrhosis morphologically and histologically similar to that induced by long-term hepatitis virus infection, which was of great clinical relevance.12–14 Our rat cirrhotic model was successfully developed via drinking 0.03% TAA water for 29 weeks and confirmed by histological evidence and quantification of hydroxyproline.

In order to avoid interference of TAA with our observation endpoints we withdrew TAA before irradiation, which was based on the following considerations. (1). In our study the intention was to investigate regeneration of the cirrhotic liver after irradiation injury for Chinese hepatocellular carcinoma with the background of HBV induced cirrhosis, which was of great clinical relevance.12–14 Our rat cirrhotic model was successfully developed via drinking 0.03% TAA water for 29 weeks and confirmed by histological evidence and quantification of hydroxyproline.

We did notice that withdrawal of TAA upon successful establishment of cirrhosis and prior to IR would result in partial resolution of cirrhotic condition in rats,15–17 but there was still hepatic injury residual up to 120 days late as demonstrated by our previous study.18 This cirrhosis resolution accompanied with liver function recovery is really different from the clinical condition, in which the cirrhosis is under progression, especially for HBV induced cirrhosis. However, we could not find an animal model, in which cirrhosis is persistent after withdrawal of hepatic toxin.

In the current study partial resolution was indeed noted among the control group (0–0 Gy) after TAA withdrawal, as demonstrated by histologically attenuation of collagen fiber and decrease of TGF-β1, less prominent trichrome stain and hydroxyproline content, but cirrhosis persisted throughout the observation period. Another evident change was significant deceleration of liver proliferation after TAA withdrawal. It was postulated that liver regeneration, stimulated by TAA-induced hepatic injury, decelerated because of the
withdrawal of regeneration stimulant. Therefore, the kinetic changes of cirrhosis and regeneration in control rats after TAA withdrawal would be the baseline and the interpretation of outcomes after irradiation should always be based on comparison with that in control group.

There was no golden standard for the determination of liver regeneration but it was proposed that at least two methods should be used. In our study PCNA-LI along with other regeneration parameters, LPI, MI, LI were used. Serum growth factors, HGF, VEGF, TGF-α and IL-6 were also assessed. In Experiment 1, IR dose of 5 Gy to 15 Gy had been confirmed to induce hepatic injury and trigger proliferation of both the right- and left- half liver, with more significant regeneration noted in the higher-doses-irradiated liver, even though insignificant changes was noted for serum TGF-α and IL-6 compared with that of the control.

Experiment 2 also confirmed that 15-Gy for induction of right-half liver injury could truly stimulate proliferation of the low-dose irradiated left-half liver, as presented in Experiment 2 with higher LPI, PCNA-LI, MI, LI and serum HGF, VEGF. IL-6 noted, compared with those of the controls. The changes of IL-6 were intriguing, which maintained stable after half-liver IR and rose remarkably with both left and right halves liver IR. The probable explanation was that as a regeneration and inflammatory factor IL-6 up-regulated by more serious IR-induced hepatic injury. Obvious tendency for more enhanced regeneration with higher doses was also seen in the left-half liver. Similar to that noted in Experiment 1, regeneration was also seen in the right-half liver. From 2 experiments, IR to both-halves of the liver comparatively resulted in more severe hepatic injury, which may have induced a more prominent regeneration in irradiated both-halves of the liver.

TGF-β1 was reported to play a key role in fibrosis including IR-related lung and liver fibrosis as well as liver regeneration. Increase of TGF-β1 paralleled liver regeneration process and peaked at the end of regeneration, acting as a key negative regulator. In Experiment 1, increased expression of TGF-β1 was noted not only in both serum and in situ in the irradiated liver, but also in the unirradiated liver. In Experiment 2, TGF-β1 increased even more significantly. Our results demonstrated that TGF-β1 not only played a role in hepatic fibrosis, but also in regeneration because TGF-β1 IHC index was also increased in the unirradiated liver. However, the role of TGF-β1 merits further investigation.

The results from the current study supported our measurements adopted in IR planning for HCC associated with cirrhosis to protect partial normal liver tissue from IR. The protected cirrhotic liver was able to regenerate to compensate the lost hepatic function during and after IR. However, we did find that higher dose resulted in more severe hepatic injury and triggered stronger regeneration, but it occurred in IR doses used in this study. We expected there should be IR dose thresholds, over which irradiated live lost the capability of repopulation. In clinical practice it is important to find the threshold, and then we will keep the normal liver dose in appropriate level.

In summary, for cirrhotic liver triggered by partial liver IR unirradiated and low dose-irradiated liver (5–15 Gy) could regenerate, and moreover, higher IR dose resulted in worse liver dysfunction and triggered a more enhanced regeneration.

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