Acute Effects of Fast Neutron Irradiation on Mouse Liver

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Fast neutrons/Liver injury/CYP2E1/TGF-β1.

Until now, the multiple biological effects of ionizing radiation on liver have been reported. However, there has not been any reports of fast neutron-mediated liver injuries including liver regeneration or fibrosis. Here, we described the biological effects of acute fast neutron irradiation on the liver. After the fast neutron irradiation of 0, 0.25, 1, 2, 4 and 8 Gy on mice, hepatocyte necrosis and a decrease in the total number of hepatocytes were induced dose-dependently. Binucleated hepatocytes and PCNA positive hepatocytes increased significantly at 0.25 and 1 Gy, but decreased markedly at 2, 4 and 8 Gy. The expression of cytochrome P450 2E1 (CYP2E1) showed a dose-dependent increase after fast neutron irradiation. The activation of p-Smad2/3, signaling intermediates of transforming growth factor-beta (TGF-β1), increased in hepatocytes after exposure of 0.25, 1, and 2 Gy of fast neutrons, but it was not detected in hepatic stellate cells (HSCs). In conclusion, fast neutron-induced liver damages, likely loss of hepatocytes, necrotic foci and vacuolar changes, were noted on the dose dependent manner and hepatocellular regeneration were significantly diminished at doses of 2, 4 and 8Gy in a dose-dependent manner. These alterations may at least in part be associated with dose-dependent increase in CYP2E1 and p-Smad2/3. These results show promise as an approach for the treatment of fast neutrons on liver tumors and in the study of pathogenesis regarding the fast neutron-irradiated damages of the liver.

INTRODUCTION

Hepatic damage by ionizing radiation, such as X-rays, γ-rays, and ultraviolet rays, have been extensively and thoroughly investigated.¹ The principle gross symptoms are hepatomegaly, hyperemia, jaundice, and ascites. The histopathology of liver specimens has shown hemorrhage extravasation, parenchymal cell loss, formation of canaliculi, fibrosis, and necrosis when whole or most of the liver was exposed to ionizing radiation.²³ In general, liver tissue has metabolic enzymes such as cytochrome P450s and several survival molecules after irradiation.¹ However, only a few specific markers of hepatic damage, have been studied. So far, major studies regarding the effect of fast neutron have focused on intestinal and neuronal changes,⁴⁵ and little study has been reported on fast neutron-induced hepatic injuries including hepatocyte proliferation and fibrosis etc.

Ionizing radiation is known to generate reactive oxygen species (ROS) in irradiated tissue and cells.⁶⁷ To control the flux of ROS, aerobic cells in liver tissue have developed their own defense system, the antioxidant system, which includes enzymatic and non-enzymatic components.⁷ Thus, liver tissue has been noticed to have an enhanced utilization of the antioxidant system to detoxify free radicals generated by radiation. Among the subcellular organelles, the mitochondria are major sites of oxidative damage, whose alteration leads to various cytotoxic effects including cell death, and mitochondrial damage associated with cytochrome P450 2E1 (CYP2E1), after the treatment of ionizing irradiation in liver of rat.¹³

In particular, ionizing radiation is one of the few exogenous agents known to cause latent transforming growth factor-beta 1 (TGF-β1) expression in DNA-damaged lesions.⁸⁹ Accumulated evidence suggests that TGF-β1 and platelet-derived growth factor are the most important cytokines responsible for the activation and proliferation of hepatic stellate cells (HSCs) in the liver. It is believed that TGF-β1 plays a key role in the transformation and collagen produc-
tion of HSCs thus leading to induction of liver fibrosis.\textsuperscript{10–12)}

Fast neutrons have been considered inappropriate for cancer therapy due to their many hazardous effects in normal organs. However, the combination therapy of fast and boron neutron capture is currently being studied as a possible treatment.\textsuperscript{13)} Girod \textit{et al} reported that the mixed irradiation of fast neutrons and gamma rays showed a synergic effect in cancer cell therapy.\textsuperscript{14)} Therefore, we investigated the dose-dependent effects of fast neutron irradiation in the livers of mice.

**MATERIALS AND METHODS**

**Experimental Design**

Male, Balb/c mice (n = 30, 7–8 weeks of age) were purchased from the Jackson Lab. (Bar Harbor, Maine, USA). The animals were housed, five to a cage, in conventional animal facilities with NIH-07 diet and water \textit{ad libitum} under constant temperature (23 ± 1°C) and a 12hr light and dark cycle. Irradiation was performed on Balb/c mice as described previously.\textsuperscript{5)} Briefly, all animals were situated in close-fitting Perspex boxes (22 × 11 × 4 cm) and irradiated by fast neutron energy generated by a cyclotron (MC-50, Scanditronix, Sweden). The conditions for irradiation induced neutron energy of 50 Mev; the rate of irradiation, 30cGy/min, and flatness is within ±3% of the dose in the field of irradiation. Groups of five mice were exposed to doses of fast neutrons, doses of 0, 0.25, 1, 2, 4, and 8 Gy. We sacrificed mice at 6 hr after irradiation in order to observe histological changes by irradiation because biological damage was time-dependent and maximal at 6hr in our previous study.\textsuperscript{5)} All animals were treated in accordance with the National Institute of Health’s \textit{Guide For the Care and Use of Laboratory Animals}.

**Histopathological and Immunohistochemistical Analysis**

From each mouse, four pieces of the liver were rapidly removed and fixed in a 10% neutral buffered formalin, processed routinely, and embedded in paraffin. The organs were cut into 4 μm sections and stained with hematoxylin and eosin (H&E). A histopathological grading of hepatic lesions was quantified by conventional Histology Activity Index (HAI) scores.\textsuperscript{15)} For immunohistochemistry, sections were deparaffinized in xylene, incubated in a solution of 3% H\textsubscript{2}O\textsubscript{2} methanol for 30 min, and microwaved at 750W for 10 minutes in a 10mmol/L citrate buffer (pH 6.0). Sections were then washed with a phosphate-buffered saline (PBS), and immunostained with antibodies of proliferating cell nuclear antigen (PCNA), phospho-Smad2/3 (p-Smad2/3) (SantaCruz Biotechnology Inc., U.S.A.), alpha-smooth muscle actin (α-SMA) (Sigma Co., U.S.A.), and CYP2E1 (Chemicon International, Inc., U.S.A.). The antigen-antibody complex was visualized by an avidin-biotin-peroxidase complex solution using an ABC kit (Vector Laboratories, USA) with 3,3-diaminobenzidine (Zymed Laboratories Inc.,

**Table 1.** Dose-dependent hepatic lesions and rates of hepatocyte fluctuation

<table>
<thead>
<tr>
<th>Dose (Gy)</th>
<th>Hepatic lesion (Score a)</th>
<th>Increase or Decrease Rate (%)</th>
<th>(No. of Hepatocytes)b</th>
<th>(No. of Binucleated Hepatocytes)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>None (0)</td>
<td>100%</td>
<td>(306.3 ± 2.2)</td>
<td>(30.0 ± 2.2)</td>
</tr>
<tr>
<td>0.25</td>
<td>Mild (0.8 ± 0.45)</td>
<td>94.1%</td>
<td>(288.2 ± 9.0)</td>
<td>(43.0 ± 1.4)</td>
</tr>
<tr>
<td>1</td>
<td>Mild (1.2 ± 0.45)</td>
<td>88.9%</td>
<td>(272.1 ± 9.0)</td>
<td>(40.5 ± 3.1)</td>
</tr>
<tr>
<td>2</td>
<td>Mild (1.2 ± 0.45)</td>
<td>85.6%</td>
<td>(262.1 ± 12.6)</td>
<td>(23.8 ± 1.5)</td>
</tr>
<tr>
<td>4</td>
<td>Moderate (2.8 ± 0.45)</td>
<td>82.4%</td>
<td>(251.8 ± 17.2)</td>
<td>(23.0 ± 0.8)</td>
</tr>
<tr>
<td>8</td>
<td>Moderate (3 ± 0.71)</td>
<td>79.1%</td>
<td>(241.8 ± 9.4)</td>
<td>(17.3 ± 1.9)</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Histopathological grades of hepatic lesions were quantified by conventional Histology Activity Index (HAI) scores as outlined in Materials and Methods.

\textsuperscript{b}Hepatocytes were counted from five different central vein areas from each mouse at a × 66 magnification per section. All data were expressed as means ± SD.
USA). They were then rinsed in distilled water and counterstained with Mayer’s hematoxylin or methyl green.

**Cell Counting and Statistics**

To determine the cell proliferation rate, we measured countable hepatocytes and binucleated hepatocytes in five randomly-selected different central vein areas from each mouse, at a magnification of $\times 66$ per section, using the image analysis system (Matrox Graphics Inc., Canada). All data were expressed as means $\pm$ SD and statistical analyses were performed by a nonparametric Mann-Whitney U test using SPSS for Windows (Release 12.0.1, SPSS Inc., USA). The level of statistical significance was set at $P < 0.05$.

**RESULTS**

**Dose-dependent Hepatic Lesions After Fast Neutron Exposure**

Table 1 shows dose-dependent hepatic lesions and the rate of increase or decrease of the total hepatocytes and binucleated hepatocytes after fast-neutron irradiation. At 0.25 and 1 Gy, there was a small loss of hepatocytes (below 11.1%) but there was an increase in binucleated hepatocytes (Table 1). From 2 to 8 Gy, a moderate loss of hepatocytes (14.4–20.9%) was observed consequently leading to hepatic cord destruction, and the binucleated hepatocytes markedly decreased. Multi-focal necrotic foci and inflammatory reactions were observed from 1 Gy irradiation (Fig. 1A). The number and size of the necrotic foci increased depending on the radiation dose. In particular, mild microvesicular fatty changes were detected mainly in zones 1 or 2 of the liver that received 8 Gy (Fig. 1B), but apoptotic bodies were rarely observed regardless of the dose. In order to find a further dose-dependent relationship after fast neutron irradiation, the total numbers of hepatocytes per field or binucleated hepatocytes were counted using image analysis (Fig. 1C and D). The total number of hepatocytes significantly decreased compared to the control group (0 Gy) after different doses

![Fig. 1. Hepatic injuries and the total number of hepatocytes after fast neutron irradiation.](image_url)
of fast neutron irradiation (Fig. 1C). Fast neutron irradiation resulted in the dose-dependent degeneration of hepatocytes, such as vacuolar degeneration and focal necrosis. The number of binucleated hepatocytes increased at 0.25 and 1 Gy compared to the control group. This increased pattern is hypothesized to be a compensatory response to the loss of hepatocytes (Fig. 1D), however, there were less binucleated hepatocytes between 2 and 8 Gy of irradiation, even though a marked loss of hepatocytes occurred.

**Immunohistochemical Changes After Fast Neutron Exposure**

To determine changes in hepatocyte proliferation, we investigated the changes of PCNA in the liver. In immunohistochemistry, PCNA-positive cells significantly increased in the liver section after 0.25 and 1 Gy of irradiation, but decreased at 4 and 8 Gy (Fig. 2) as compared to the non-irradiated control group. The expression of CYP2E1 increased in the hepatocytes around the central veins depending upon

![Fig. 2](image_url)
Effects of Fast Neutron on Mouse Liver

The expression of CYP2E1 in the mouse liver after fast neutron irradiation. A: The expression of CYP2E1 increased in the hepatocytes around the central veins (CV), depending on the doses of irradiation and the exp. peaked at 8 Gy. Immunostaining for CYP2E1. Original magnification (×66). B: The number of immunopositive cells for CYP2E1 antibody was counted in ten fields. Each bar represents the means ± SD of cell numbers. *P < 0.05 versus non-irradiated group (0 Gy) (Mann-Whitney U test using SPSS program).

DISCUSSION

In general, the liver is exposed as normal tissue during radiation therapy for tumors in the upper abdomen or lower thorax region, however, therapeutic exposure can lead to...
Fig. 4. The activation of p-Smad2/3 in the liver of mice after fast neutron irradiation. A: The p-Smad2/3 is extensively expressed in the hepatocytes at 0.25 and 1 Gy irradiation, and the amount peaks at 2 Gy. There is little expression of p-Smad2/3 at 4 and 8 Gy. Immunostaining for p-Smad2/3. Original magnification (×132). B: The number of immunopositive cells for p-Smad2/3 antibody was counted in ten fields. Each bar represents the means ± SD of cell numbers. *P < 0.05 versus non-irradiated group (0 Gy) (Mann-Whitney U test using SPSS program). C: There was no immunoreactivity for α-SMA from any doses of the irradiated liver sections. Immunostaining for α-SMA. Original magnification (×66).
radiation hepatitis, fibrosis, and sometimes death.\(^{2,3,16}\) In the present study, there were signs of necrosis and fatty changes in the hepatocytes, including the repression of PCNA, after doses of 2, 4, and 8 Gy of fast neutron irradiation, which resulted in the decrease of the total number of hepatocytes and liver mass. Multiple biological effects can be induced by ionizing radiation through the dysfunction of cellular organelles, direct action on nucleic acids, and the production of free radical species from water.\(^{17,18}\) At 0.25 and 1 Gy of irradiation, the increase in abnormal and binucleated hepatocytes indicates severe liver damage by radiation-generated free radicals. In general, an increase in hepatocyte size and in the number of binucleated hepatocytes have been described in age-related changes of hepatic structure.\(^{19}\) However, under pathologic status such as irradiation-mediated damage, binucleated cells mean a fusion of two abnormal hepatocytes by radiation-generated free radicals or regeneration against liver damage.\(^{20}\) To distinguish between fusion and regeneration in the liver, we used PCNA staining. Finally, we found positive correlation of binucleated hepatocytes and proliferating hepatocytes against fast-neutron-mediated liver damage (Fig. 2). The number of binucleated hepatocytes and PCNA positive hepatocytes decreased at 2, 4 and 8 Gy of fast neutron irradiation, suggesting that liver regeneration diminished. Geraci et al.\(^{14}\) also reported that \(\gamma\)-radiation (5 Gy) inhibited growth of the hepatocytes obtained between 85 and 90 days after irradiation, and that radiation doses of more than 10 Gy resulted in a dose-dependent decrease in the liver parenchymal cell population.

During irradiation, radiation-generated ROS attacked membrane phospholipids and cholesterol esters, causing lipid peroxidation and fatty change.\(^{2,7,17}\) Several studies have shown that the expression of cytochrome P450 is highly associated with oxidative stress, caused by hydroxyl free radicals. Free radicals are one of the important radiation-induced free radical species, and can lead to cell injury.\(^{1,6,20}\) Chung et al.\(^{1}\) reported that \(\gamma\)-ray irradiation at the exposure level which can induce organelle dysfunction-induced CYP2E1 in the liver, might be associated with mitochondrial damage. In the present study, we observed microvesicular fatty change in hepatocytes after 8 Gy of fast neutron irradiation and a dose-dependent increase of CYP2E1 expression after fast neutron irradiation. These signs indicated that generation of ROS could cause cellular membrane damage or induce CYP2E1 expression.

Recent studies have demonstrated that TGF-\(\beta\), which is usually secreted in a latent form and is engulfed by neighboring cells,\(^ {21}\) is activated rapidly in an \(\gamma\)-ray irradiated mouse mammary gland and in the liver of rats in vivo.\(^ {9,22}\) The conversion of TGF-\(\beta\), from a latent to an active form, may be one of the earliest in vivo responses to low doses of radiation in mice.\(^ {9,22,23}\) However, until now, there have been no reports regarding the activation of latent TGF-\(\beta\) by fast neutron irradiation. In general, the activation of latent TGF-\(\beta\) has been demonstrated by the activation of p-Smad2/3, signaling intermediates of active TGF-\(\beta\).\(^ {10,22}\) In our study, the activation of p-Smad2/3 was observed in hepatocytes which continued with other study.\(^ {22}\) This suggest that only hepatocytes were a major target cell of active TGF-\(\beta\) after fast neutron irradiation between 0.25 ~ 2 Gy and that only low doses of fast neutron might activate latent TGF-\(\beta\). This is because p-Smad2/3 activation was not detected at high doses of irradiation. It is believed that quiescent HSCs were transformed into fibrogenic myofibroblast producing collagen fibers by TGB-\(\beta\).\(^ {10-12}\) These myofibroblasts usually express \(\alpha\)-SMA, a specific marker of activated HSCs, in their cytoplasm.\(^ {24,25}\) however, there were no positive HSCs for p-Smad2/3 and \(\alpha\)-SMA nor fibrosis in this study.

Since several radiotherapy techniques have been developed,\(^ {26}\) interest in radiotherapy as a treatment for liver tumors has been increasing among oncologists.\(^ {27}\) While the radiobiological effects on tumors has been extensively investigated for X-rays and \(\gamma\)-rays, little work has been directed toward fast neutrons. Fast neutrons had been considered inappropriate for the cancer therapy due to their many hazardous effects in intestinal walls. Therefore, the combination of therapies of fast and boron neutron capture are currently being studied as possible treatments.\(^ {13}\) Therefore, better knowledge of the mechanisms involved in the cellular responses of fast neutrons is particularly important as a promising approach for the treatment of cancer and in the study of pathogenesis regarding fast neutron-irradiated damage to solid organ.

In conclusion, there were signs of dose-dependent hepatic damage such as necrosis of hepatocytes, reduction of proliferation, and fatty change, found histopathologically in the present study. It is interesting that the expressions of CYP2E1 increased in a dose-dependent manner after fast neutron irradiation. In addition, the expression of p-Smad2/3, activated by latent TGF-\(\beta\), increased after low doses of fast neutron irradiation, but there was no activation of HSCs nor any evidence of fibrosis. Further studies on the mechanisms of hepatic enzyme responses against fast neutron irradiation remains to be resolved.

**ACKNOWLEDGMENTS**

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