J. RADIAT. RES., 41, 151–161 (2000)

Relative Biological Effectiveness of Carbon Ions for Causing Fatal Liver Failure after Partial Hepatectomy in Mice

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(*Received, November 4, 1999*)
(*Revision received, June 12, 2000*)
(*Accepted, June 13, 2000*)

Lethal dose 50/Hepatic failure/Carbon ions/Mitosis/DNA synthesis

To evaluate the acute phase damage to liver by carbon ions, BALB/c mice were irradiated with carbon ions or X-rays after two-thirds partial hepatectomy, and their survival was followed. The 50% lethal dose within 60 days ($LD_{50/60}$) was 42.2 ± 0.25 Gy (standard error) for X-rays, and 22.7 ± 0.25 Gy for carbon ions. The relative biological effectiveness (RBE) of carbon ions was 1.86 (95% confident limits: 1.69–2.04) as calculated from the $LD_{50/60}$. Mice irradiated at much higher doses, 60 Gy of X-rays or 24 Gy of carbon ions, showed significantly higher serum ammonia levels and lower serum albumin levels than normal, suggesting hepatic failure as a cause of death. Hepatocytes showed karyorrhexis and karyolysis in carbon ion irradiated and spotty necrosis in X-ray irradiated mice, suggesting nuclear damage. Mice irradiated with LD_{50} of X-rays or carbon ions had a remarkably lower bromodeoxyuridine (BrdU) labeling index and mitotic index than control. Treatments with both BrdU and vincristine showed that none of the hepatocytes that synthesized DNA after irradiation completed mitosis, indicating G2 arrest. The liver weight of irradiated mice significantly decreased depending on the dose. Carbon ions as well as X-rays damaged hepatocytes directly and suppressed liver regeneration leading to fatal liver failure.

INTRODUCTION

Heavy ion irradiation has stronger biological effects than X-ray irradiation[1)]. Carbon ions have been used as a source of heavy ion in clinical trials at the National Institute of Radiological Sciences in Japan (NIRS) using the Heavy Ion Medical Accelerator in Chiba (HIMAC) since June 1994[2)]. In June 1995, clinical trials in patients with hepatocellular carcinoma

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(HCC) started because carbon ions are expected to be effective against human HCC, a major cause of cancer death in Japan[3].

Current clinical trials of carbon ions to treat HCC use fractionated extracorporal irradiation. In the future, we will begin another trial with carbon ions, total liver irradiation to prevent the growth of small metastasis of HCC after resection of HCC and surrounding liver tissue. If liver regeneration is insufficient, the patients' condition will become critical. Information on liver regeneration and acute liver damage caused by carbon ions is needed.

Chronic irradiation liver damage manifests as liver fibrosis disrupting the blood circulation in rodents[4]. In humans, irradiation hepatitis is reported as a form of chronic liver damage[5]. Acute damage by irradiation, however, has not been reported in either animals or humans. Chromosomal damage is attributed to the injurying to liver caused by X-rays as well as high linear energy transfer (LET) radiation, such as neutron irradiation[6–8]. Likewise, heavy ions cause chromosome breakages and gene mutations that are fatal to cells, sometimes leading to cancer[3,9,10]. No report exists, however, on the survival of mice as well as liver function which is directly relevant to clinical trials for HCC.

The liver consists of reverting postmitotic hepatocytes, and is relatively resistant to irradiation[11]. There are several reports of irradiation damage to liver following X-ray treatment[5,12]. X-rays cause G2 arrest following irradiation, allowing for the possible repair of DNA damage[13]. It is thought that X-rays or carbon ions suppress liver regeneration and DNA synthesis leading to liver failure after partial hepatectomy. Liver failure is accompanied by hypoalbuminemia and hyperammonemia[14,15]. Although clinically important, the suppression of liver regeneration and liver failure has not been reported on.

As a first step to evaluating the biological hazard of carbon ions, we analyzed the relative biological effectiveness (RBE) of carbon ions for fatal hepatic failure. The RBE of carbon ions has been reported with several radiological endpoints such as cell survival in mouse intestine, skin reaction of mouse and killing and transformation of Syrian hamster embryo cells[16–18]. The RBE of neutrons for liver damage was reported by Ono et al[6]. The timing of irradiation in this study is different from that to be used in our clinical trials for HCC; they irradiated before partial hepatectomy. Therefore, the RBE data is of limited use for our future trials. In the present study, we irradiated the remnant liver with X-rays or carbon ions after partial hepatectomy as a model of post surgery irradiation of HCC to clarify acute irradiation damage essential for clinical trials[19].

MATERIALS AND METHODS

Animals

Male BALB/c mice were produced at the National Institute of Radiological Sciences (NIRS) (Chiba, Japan) under specific pathogen free (SPF) conditions. At the age of 6 weeks and weighing 20–25 grams, they were moved to and housed in a conventional room of the animal research facility at NIRS. They were provided food and acidified water *ad libitum*, and maintained in a consistent light-dark cycle (lights on : 7 am, off: 7 pm) both before and after

the experimental procedures. Mice were sacrificed with carbon dioxide according to the guidelines of the NIRS animal use committee.

Surgery and irradiation

A two-thirds partial hepatectomy was performed under anesthesia with an intraperitoneal injection of pentobarbital (50 mg/kg body weight)[20].

The design of the irradiation setting is depicted in Fig. 1. Since the liver is surrounded by radiosensitive tissues, it was necessary to shield them from irradiation. The stomach and intestine were moved out of the radiation field using aseptic techniques. The remnant liver was irradiated immediately after partial hepatectomy with X-rays through a slit in the shield box, or with carbon ions through a slit in the collimator. The slit was shaped like the remnant liver, but was slightly larger in order to cover the whole remnant liver during inspiration and expiration. The position of the remnant liver was determined by referring to roentgenographs taken after painting an operated liver with contrast medium. The spleen was always shielded because it was on the left side of the abdominal cavity and the remnant liver was on the right. Throughout the operation and irradiation procedures, great care was taken to keep the surface of the liver, intestine, stomach, and spleen moist by covering with gauze soaked in physiological saline to avoid bruising. The irradiation field was visualized through the slit in the shield

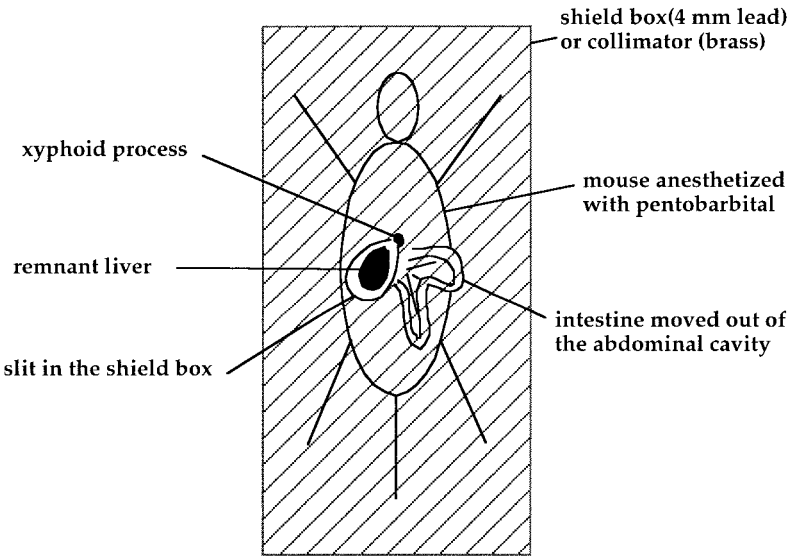

Fig. 1. Irradiation of remnant liver alone after partial hepatectomy. The remnant liver was irradiated with carbon ions or X-rays after two-thirds partial hepatectomy. The shield box (for X-ray) or the collimator (for carbon ions) was set so that the top of the slit was right above the xyphoid process. The slit always covered the entire remnant liver from inhalation through exhalation. The intestine and the stomach were moved out of the abdominal cavity to protect them from irradiation, and covered with gauze soaked in physiological saline. The spleen was always shielded because it was on the left side of the abdominal cavity and the remnant liver was on the right.

box for every mouse to confirm that the stomach, intestine and spleen were out of the field. The irradiation was completed within 1 hour after partial hepatectomy, and the abdomen was closed. The abdominal cavities of unirradiated control mice were kept open for 1 h and closed.

Groups of 6 mice received 30, 35, 40, or 45 Gy of X-rays in a single exposure (groups 30X, 35X, 40X, and 45X, respectively). Sixteen mice received 50 Gy and ten mice received 60 Gy of X-rays (groups 50X and 60X, respectively). Groups of four mice received 19, 20, 21, 22, or 23 Gy of carbon ions (groups 19C, 20C, 21C, 22C, and 23C, respectively), and a group of 10 mice received 24 Gy (group 24C). A control of 5 mice underwent partial hepatectomy but were not irradiated.

After irradiation or sham operation, the survival of mice was observed. When they died, liver weight was measured as an indicator of the amount of functioning hepatocytes. The mortality rate of mice was calculated as: mortality rate $\pm$ standard deviation $(\mathrm{SD}) = \mathrm{S}/\mathrm{N} \pm \sqrt{\mathrm{S}(1-\mathrm{S}/\mathrm{N})/\mathrm{N}}$, where S is the number of dead mice within 60 days after irradiation and N is the number of total irradiated mice, and standard error $(\mathrm{SE}) = \mathrm{SD}/\sqrt{\mathrm{N}}$. The irradiation dose at which 50% of mice were dead was defined as LD_{50}.

Sources of irradiation and dosimetry

X-rays were generated at 200 kVp/20 mA and filtered through 0.5 mm of Cu and Al (Pantak HF-3205, Pantak, Inc., Branford, CT) at a dose rate of 1.20 Gy/min. The shield box was made of lead 4 mm thick, which reduced the irradiated dose to less than 1/1000. Dose measurements were made with a Victoreen Condenser R-meter placed in the position of the remnant liver of each mouse.

For accelerated carbon ion irradiation, methods of Ando et al were followed with slight modification[17]. Briefly, carbon ions were generated by the HIMAC synchrotron at 290 MeV/u. An ionizing-chamber method was adapted for the dosimetry of carbon ions[21]. The remnant liver was irradiated at a dose rate of 3 Gy/min. Under the conditions used, the average LET of carbon ions was 50 $\pm$7 keV/μm at the center of liver. The irradiation field was defined by a brass collimator.

Blood analysis

Blood was drawn by cardiac puncture from the left ventricle under anesthesia with pentobarbital 10 days after irradiation in group 60X (3 mice), group 24C (3 mice), and unirradiated controls (group Co, 6 mice). Serum protein and serum albumin were analyzed. Serum ammonia was also examined in group 60X(9 mice), group 24C (7 mice), and group Co (5 mice). Analyses were performed by the Special Reference Laboratory (Tokyo, Japan). Also liver weight was measured to compare with blood analysis.

Histological examination

Three mice were sacrificed for histological analysis 10 days after irradiation with 22.7 Gy of carbon ions (22.7C), irradiation with 42.2 Gy of X-rays after partial hepatectomy (42.2X), or no irradiation as a control (Co). These radiation doses correspond to the LD_{50} of

carbon ions and X-rays, respectively, as shown in the result section. Representative tissues from all organs were removed, fixed in 10% formalin, and processed routinely for histological examination.

BrdU index and mitotic index

Mice were divided into three groups (4 mice per group) : non-irradiated as control, and irradiated with 42.2 Gy of X-rays, or 22.7 Gy of carbon ions. Mice were injected with BrdU (Wako Pure Chemical Industries, Ltd, Tokyo, Japan) intraperitoneally at 50 mg/kg at a concentration of 5 mg/ml in physiological saline every 12 h after partial hepatectomy. One h after the injection, individual mice were sacrificed at 48 h after partial hepatectomy because the BrdU labeling index reached a plateau at 40 h after partial hepatectomy in all groups. Tissue samples were fixed with 4% paraformaldehyde for 24 h, embedded in paraffin, and immunostained with monoclonal BrdU antibody (Becton-Dickinson, Franklin Lakes, NJ). Liver weight was also measured. BrdU positive hepatocytes were counted from a portal space to a central vein in randomly selected fields; nuclei from 5000 hepatocytes were examined. The BrdU labeling index was calculated as the number of BrdU positive hepatocytes/1000 hepatocytes.

Mice were injected with vincristine (Wako Pure Chemical Industries, Ltd, Tokyo, Japan) intraperitoneally every 12 h after partial hepatectomy at a dose of 0.25 mg/kg to block the cell cycle of all hepatocytes at the M phase . Three groups of 5 mice were examined: irradiated with 42.2 Gy of X-rays or 22.7 Gy of carbon ions, or not irradiated (control). The mitotic index reached a plateau at 48 h after partial hepatectomy in non-irradiated control, and at 70 h in mice irradiated with 42.2 Gy of X-rays or 22.7 Gy of carbon ions (data not shown). Mice were sacrificed 70 h after partial hepatectomy, the liver was weighed as well as subjected to H&E staining for determining the accumulated mitotic index. Mitotic hepatocytes were counted from a portal space to a central vein in randomly selected fields; nuclei from 5000 hepatocytes were examined. The mitotic index was calculated as the number of mitotic hepatocytes/1000 hepatocytes.

Combined treatments with BrdU and vincristine

The percentage of hepatocytes labeled with BrdU progressing from S phase to M phase of the cell cycle was determined. BrdU was injected intraperitoneally at 50 mg/kg, 24 h after partial hepatectomy, and vincristine was injected intraperitoneally at the dose of 0.25 mg/kg every 12 h beginning at 24 h after partial hepatectomy. Mice were sacrificed 70 h after partial hepatectomy, and the liver was weighed and then immunostained for BrdU. BrdU staining was performed as reported elsewhere[22]. All BrdU-labeled hepatocytes as well as mitotic hepatocytes labeled with BrdU were counted, and the percentage of labeled hepatocytes that were mitotic was calculated. Mice were divided into three groups : non-irradiated as control (5 mice), irradiated with 42.2 Gy of X-rays (5 mice), and irradiated with 22.7 Gy of carbon ions (5 mice) after partial hepatectomy. We compared the BrdU labeling index and mitotic index of mice irradiated with the $LD_{50/60}$ of X-rays and carbon ions and chose 42.2 Gy for X-rays and 22.7 Gy for carbon ions.

Statistical analysis

Probit analysis was applied to calculate the 50% lethal dose within 60 days ($LD_{50/60}$). The Kruskal-Wallis test was applied to test statistically significant differences between experimental groups. A 5% significance level was accepted as the criterion for statistical significance.

RESULTS

Mortality rate and RBE

We observed the survival of mice until 90 days after irradiation, and found that no mice died later than 27 days (data not shown). We concluded that death due to acute irradiation reached a plateau in mice irradiated with X-rays or carbon ions at 60 days after irradiation.

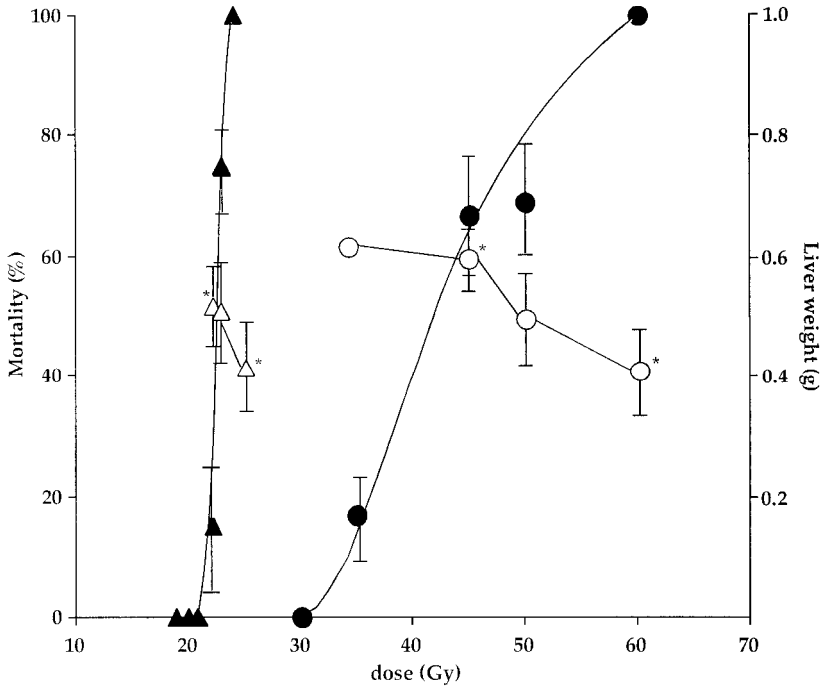

Fig. 2. Mortality rate and liver weight of mice irradiated with X-rays or carbon ions. Mortality rates were determined at 60 days after irradiation by using the equation described in text.
* Mean liver weight were lower for 60X and 24C groups than 45X and 23C groups , respectively. Numbers of mice irradiated with X-rays were 6 (30 Gy, 35 Gy, 40Gy, 45 Gy), 10 (60 Gy), 16 (50 Gy), respectively. Those of mice irradiated with carbon ions were 4 (19 Gy, 20 Gy, and 21 Gy), 8 (23 Gy), 10 (24 Gy) and 13 (22 Gy), respectively (see text). Liver weight in mice irradiated with X-rays ; numbers were 1 (35 Gy), 4 (45 Gy), 11 (50 Gy), 10 (60 Gy), respectively. Liver weight in mice irradiated with carbon ions ; numbers were 2 (22 Gy), 6 (23 Gy), 10 (24 Gy), respectively. Error bars denote standard errors. ● : mortality rate of mice irradiated with X-rays, ▲ : mortality rate of mice irradiated with carbon ions, ○ : liver weight of mice irradiated with X-rays, △ : liver weight of mice irradiated with carbon ions.

The mortality rates of mice dead within 60 days after irradiation with different doses of X-rays and carbon ions were obtained using the formula described earlier and are shown in Fig. 2.

The mortality curve for carbon ions was steeper than that for X-rays. The $LD_{50/60}$ of X-rays was 42.2 ± 1.1(standard error) Gy, while that of carbon ions was 22.7 ± 0.3 Gy according to Probit analysis. RBE was then determined to be 1.86 (95% confident limits: 1.69–2.04).

Mean liver weight of 60X and 24C groups were significantly lower than those of 45 X and 22C groups, respectively ($p < 0.05$). Liver weight tended to decrease according to the dose of irradiation.

Blood Analysis

Peripheral blood analysis showed impaired liver function (Table 1). Total protein and albumin of moribund mice irradiated with 60 Gy of X-rays or 24 Gy of carbon ions were significantly lower than those of non-irradiated control mice 10 days after the operation ($p < 0.05$). Serum ammonia of moribund mice irradiated with 60 Gy of X-rays or 24 Gy of carbon ions were significantly higher than those of non-irradiated control mice ($p < 0.05$). Mean liver weights of the 60X and 24C groups were significantly low compared to the non-irradiated control.

Table 1. Blood analysis of mice irradiated with X-rays or carbon ions after two-thirds partial hepatectomy

	control	X-rays (60Gy)	carbon ions (24Gy)
total protein (g/dl)	4.20 ± 0.23	3.30 ± 0.56[a]	2.9 ± 0.92[a]
albumin (g/dl)	2.4 ± 0.25	1.73 ± 0.70[a]	1.10 ± 0.23[a]
serum ammonia (μg/dl)	101 ± 50	222 ± 112[a]	219 ± 103[a]
liver weight (g)	0.64 ± 0.1	0.48 ± 0.08[a]	0.47 ± 0.09[a]

Blood samples were obtained 10 days after irradiation (see text). Data are expressed as the mean ± standard error. n = 6 (control), 3 (X-ray, carbon ions) in total protein, albumin. n of serum ammonia = 5 (control), 9 (X-ray), and 7 (carbon ions).

[a] Significantly different from controls at $p < 0.05$.

Histological findings

Grossly, no remarkable changes were found in the liver or neighboring organs, such as massive necrosis or hemorrhage. Neither perforation nor bleeding of the intestine was found.

Microscopically, bone marrow was cellular and no change was found in intestinal mucosa. The liver structure was not disorganized in the 22.7C group. On the other hand, coagulation necrosis of hepatocytes was preferentially observed in 42.2X group. Hepatocytes with karyorrhexis and karryolysis, which indicate cell death, were scattered in the liver of irradiated mice. Central venous occlusion, which is a typical finding of chronic irradiation damage to the liver, was not found. Presumably, it occurs three to six months after irradiation.

BrdU labeling index and mitotic index

The BrDU labeling index and mitotic index are shown in Table 2. X-rays and carbon

Table 2. BrdU labeling index and mitotic index of liver irradiated with X-rays or carbon ions after two-thirds partial hepatectomy

	Control	X-rays 42.2 Gy	Carbon ions 22.7 Gy
BrdU labeling index (/1000nuclei)	179 ± 1.1	37.5 ± 8.5[a]	64.5 ± 1.7[a]
Mitotic index (/1000nuclei)	26 ± 6.1	0.6 ± 0.4[b]	0.6 ± 0.4[b]
Mitosis labeled with BrdU/Mitosis (%)	2.38 ± 1.06	0 ± 0[b]	0 ± 0[b]
Liver weight (g)	0.65 ± 0.11	0.57 ± 0.1[a]	0.54 ± 0.09[a]

Vincristine was injected intraperitoneally to block mitosis and BrdU to analyze DNA synthesis (see text). BrdU positive or mitotic hepatocytes were counted from a portal space to a central vein in randomly selected fields; nuclei from 5000 hepatocytes were examined. The BrdU labeling index or mitotic index was calculated as the number of BrdU positive hepatocytes/1000 hepatocytes. Data are expressed as the mean±standard error. n = 4 (BrdU labeling index), 5 (mitotic index), and 5 (mitosis labeled with BrdU).

[a] Significantly different from controls at $p < 0.05$.

[b] Significantly different from controls at $p < 0.01$

ions suppressed DNA synthesis as shown by the BrdU labeling index. Very few hepatocytes underwent mitosis (less than 0.1%). None of the hepatocytes that underwent mitosis had gone through DNA. No significant differences were seen between the iso-effect doses of X-rays and carbon ions.

DISCUSSION

RBE is a measure of the hazard of heavy ion radiation to normal organs. We irradiated remnant liver alone after two-thirds partial hepatectomy, and found that the RBE of 50 kev/μm carbon ions was 1.86 for mortality in mice. Carbon ions and X-rays caused nuclear damage and suppressed DNA synthesis as well as mitosis, causing liver dysfunction as indicated by blood analysis.

Previously, the RBE of carbon ions was reported to be 2.3 at 65 keV/μm of LET, and 1.6 at 23 keV/μm for survival of intestinal crypt cells [16]. Since RBE shows a maximum at an LET of about 100 keV/μm, our value of 1.86, was consistent with theirs[1,16]. Another reported value, 2.3, which is higher than ours, obtained at 50 keV/μm of LET, is the same as ours for mouse skin reaction[17]. The discrepancy may be due to the difference of sensitivity between skin and liver.

Liver detoxifies serum ammonia and produces various serum proteins like albumin[14]. Liver failure is frequently accompanied by hyperammonemia and hypoalbuminemia[15]. Braun et al reported that hyperammonemia is itself fatal[23]. The serum albumin level indicates the potential of protein produced in liver. Our results showed that protein synthesis in liver was severely damaged after irradiation. The hyperammonemia and hypoalbuminemia indicate that the irradiated liver lost normal function, such as detoxification and protein production, leading to lethal hepatic failure. Also our data suggested that serum protein, albumin, and serum ammonia levels were clinically appropriate parameters with which to evaluate irradiation

damage to liver.

The morphology of hepatocytes is not altered immediately after irradiation; however, occasional cells undergoing cell death can be seen, showing karyorrhexis and karyolysis[24,25]. In our experiments, hepatocytes in the 22.7C group showed karyorrhexis and karyolysis. Liver of 42.2X mice showed spotty necrosis. Both suggested direct damage to hepatocytes in the acute phase of irradiation, which is different from veno-occlusive disease, chronic irradiation damage to liver[11]. Our data suggested that carbon ions as well as X-rays damaged chromosome, leading to cell death in the acute phase[6–8].

Previously, Ono et al performed partial hepatectomy to mice after irradiation with X-rays or neutrons to obtain RBE for induction of micronuclei as an end-point[18]. We irradiated the remnant mice liver after partial hepatetomy to obtain RBE with their survival as an end-point. They used hepatectomy to observe mitotic nuclei more effectively while we used it as a model of intraoperative irradiation therapy. Interestingly, both RBEs were around 2.0 at high dose irradiation (more than 3 Gy) although the timing of irradiation and sources of high LET irradiation were different, suggesting that neutrons and carbon ions damage nuclei of hepatocytes via the same mechanism.

Carbon ions as well as X-rays suppress DNA synthesis and mitosis of hepatocytes after partial hepatectomy[26–28]. These effects occurred at sublethal doses of irradiation, indicating that even the surviving cells were significantly damaged. Both carbon ions and X-rays inhibited DNA synthesis significantly, with even greater inhibition observed for mitosis. None of the cells that synthesized DNA after irradiation completed mitosis, indicating that they had sustained significant DNA damage. These results of G2 arrest following X-ray treatment are consistent with previously reported results[13,28,29]. This is the first report of suppression of DNA synthesis in hepatocytes by carbon ions.

The data of liver weight along with the mortality rate, blood analysis, and BrdU labeling index and mitotic index suggested that the direct toxicity and suppression of liver regeneration affected the function of hepatocytes leading to fatal hepatic failure.

Further study will be necessary to unveil the mechanism of nuclear damage and suppression of DNA synthesis as well as mitosis induced by irradiation. Our next step will be an analysis of chronic irradiation damage by carbon ions in liver.

ACKNOWLEDGEMENTS

We thank Mrs. Sachiko Ishii for her technical assistance. Thanks are also due to Dr. Masao Ohto, Dr. Hiromitsu Saisho and Dr. Kozo Morita for their encouragement and useful discussions. This work was supported by a grant from the Science and Technology Agency of Japan.

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