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Induction of DNA fragmentation by total-body irradiation in murine liver

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Summary. Total-body irradiation (TBI) is an accepted modality to treat patients with disseminated tumors. The influence of the treatment on normal tissues is evaluated using mice by measuring the rate of the induction and distribution of apoptosis, as well as DNA fragmentation which occurs in the murine liver within hours of irradiation.

Unanesthetized female C3H/He mice were exposed to γ-ray TBI of 2, 7, and 20 gray (Gy) delivered from ^{60}Co at a dose rate of 114 cGy/min. Frozen sections of livers which were excised from the animals at various times after irradiation were stained by hematoxylin-eosin (H-E) to count numbers of apoptotic cells, or were examined to detect DNA fragmentation.

The percentages of apoptotic cells and length of the period during which the maximum levels of the percentages were exhibited showed a dose-dependent increase in the sections stained with H-E. No positive cells for 3'-OH ends of fragmented DNA were found in the liver before TBI, whereas positive cells were observed immediately after irradiation without dose-dependency, these positive cells returned to nearly basal levels after several hours. Positive cells were observed prior to showing apoptosis, suggesting that DNA fragmentation occurs immediately after TBI independent of apoptosis. The difference in the time courses between induction of DNA fragmentation and of apoptosis was not observed in other organs or in the samples treated with the detergent. These results suggested that the 3'-OH ends newly generated by TBI were masked by a detergent-soluble DNA-binding molecule which might be preferentially present in the murine liver.

Key words: Total-body irradiation, Murine liver, Apoptosis, DNA fragmentation

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Introduction

The radiation dose in therapy is determined based on the radiosensitivity of the tumor and the background normal tissues. For example, most hepatomas, a radiosensitive tumor, have not been treated with irradiation because hepatic epithelial cells also have high radiosensitivity (Gunderson and Willet, 1992), excluding the selected cases which can be treated with multifield irradiation using computed tomography-optimized planning. Many clinical or experimental reports concerning late hepatic injury caused by irradiation have been published (Bollinger and Inglis, 1933; Warren and Friedman, 1942; White et al., 1955; Ogata et al., 1963; Lewin and Millis, 1973; Fajardo, 1989). However, immediate changes, including DNA damage and apoptosis induced by high-dose-rate irradiation, have never been reported. Evaluating the influence of total- or half-body irradiation (HBI) on normal tissues might contribute to care for patients, since HBI is an accepted modality for treating patients with disseminated tumors (Lin and Drzymala, 1992).

Ionizing radiation, when given in small to moderate doses, greatly enhances apoptosis in certain normal tissues without producing necrosis (Kerr et al., 1994). The importance of apoptosis is becoming clear as additional reports of its occurrence in irradiated normal and tumor tissues accumulate (Stephens et al., 1991; Waters, 1992; Akagi et al, 1993; Meyn, 1993, 1994).

In this report, the induction and distribution of DNA fragmentation detected by immunohistochemical stain *in situ* were studied and compared with the induction of apoptosis, within hours of irradiation using the murine liver as a radiosensitive and relatively homologous tissue.

Materials and methods

Animals

Female 8-week-old C3H/He mice were used. They

were maintained in our specific-pathogen-free barrier colony and received humane care in compliance with our institution's guideline.

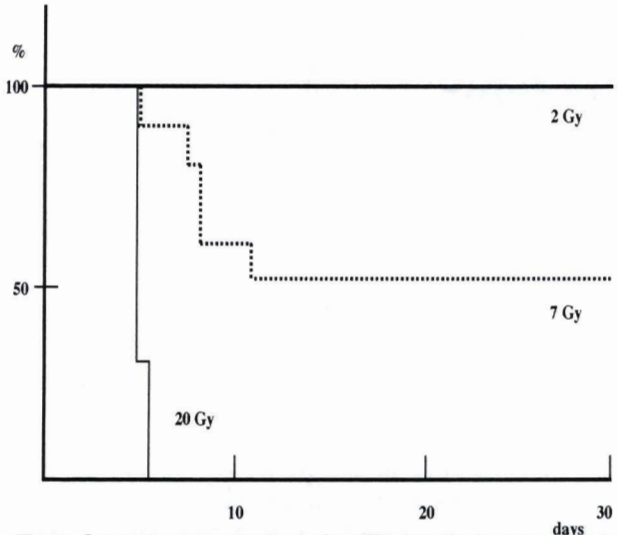

Fig. 1. Survival curves obtained after TBI with single dose of 2, 7, and 20 Gy. Ten mice of each group were observed every day.

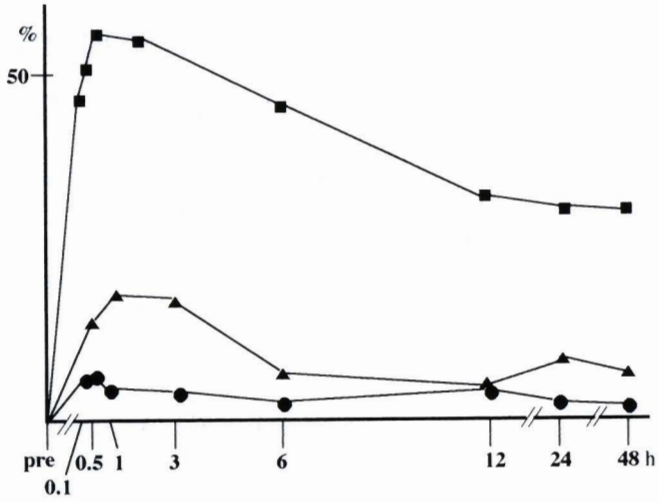

Fig. 2. Time course of induction of apoptotic cells detected using H-E stain in the livers exposed to TBI with 2 (black circle), 7 (black triangle), and 20 Gy (black square) .

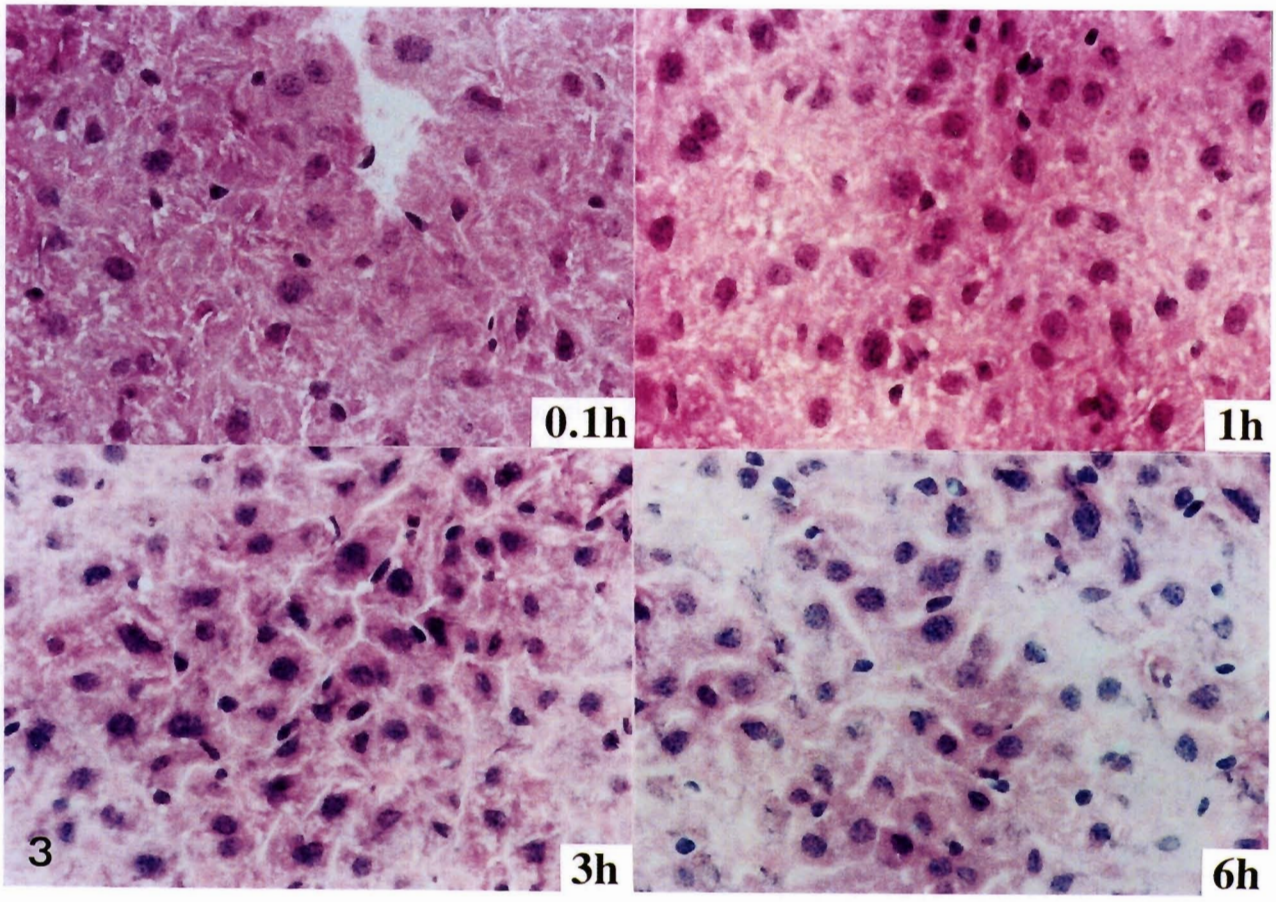

Fig. 3. Apoptosis induced by TBI of 2 Gy in H-E-stained cryosections. A few cells undergoing apoptosis exhibit condensed chromatin and nuclear fragmentation. x 400

Irradiation

Unanesthetized mice in an acrylic case were exposd to γ-ray total-body irradiation (TBI) delivered from ^{60}Co at a dose rate of 114 cGy/min.

Doses of 2, 7, and 20 Gy were employed. Twenty mice were irradiated at each dose. Ten mice were used for excision and the other mice were observed every day for 30 days after irradiation.

Measurement of cells showing apoptosis and DNA fragmentation

At pre- irradiation and 0.5, 1, 3, 6, 12, 24, and 48 hours after TBI and immediately after irradiation in the cases of 2-Gy and 20-Gy doses, mice were killed by cervical dislocation. The time immediately after TBI is represented as 0.1 hour in figures. The livers were excised immediately and stored in liquid nitrogen.

Sections of 6 μm were mounted on slides and one of them was stained with hematoxylin-eosin (H-E) for histopathological study. Cells showing condensation of chromatin as intracellular apoptotic body and aberrant nuclei were counted.

The 3'-OH ends generated by DNA fragmentation were analyzed by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) method (Gavrieli et al., 1992; Wijsman et al., 1994) using an *in situ* apoptosis detection kit (Oncor) and an *in situ* cell death detection kit (Boehringer-Mannheim). Frozen tissue sections were fixed with a freshly prepared paraformaldehyde solution. They were treated with blocking solution including H_2O_2 and thereafter with permeabilisation solution. A TdT solution was added to cover the sections, which were then incubated in a humidified chamber for 60 minutes at 37 °C. They were finally stained with diamino-benzidine and counterstained with methyl green. Some sections were treated with 10 mM 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1propane-sulfonate (CHAPS) and washed well with phosphate-buffered saline. The sections stained to detect DNA fragmentation and those stained with H-E were treated with CHAPS before permeabilisation and fixation,

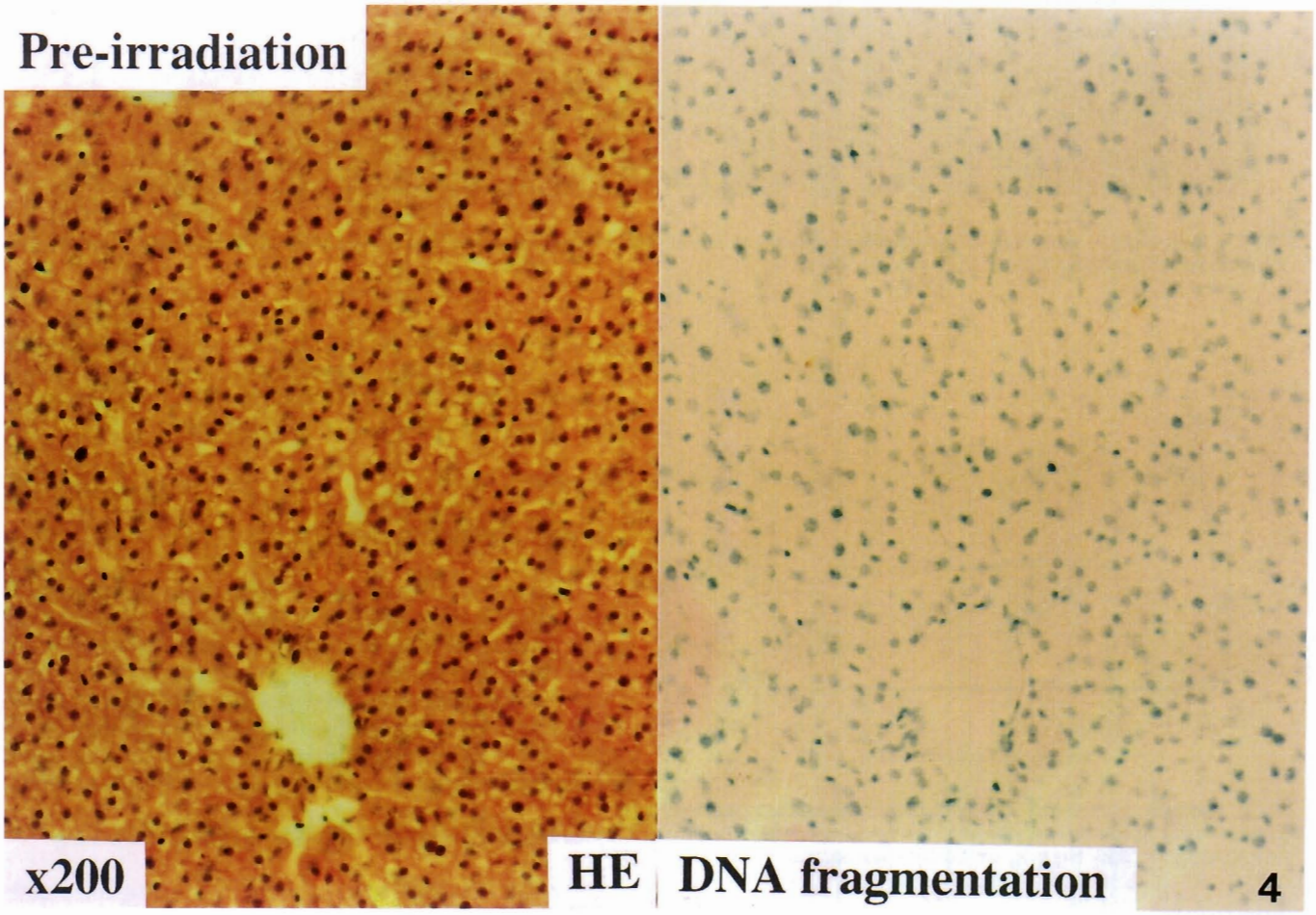

Fig. 4. Hepatic lobules in pre-irradiated mice. Tissue sections were stained with H-E (left) and immunohistochemically to detect DNA fragmentation (right). No positive cells for DNA fragmentation in lobules are seen in this methyl green-counterstained section. x 200

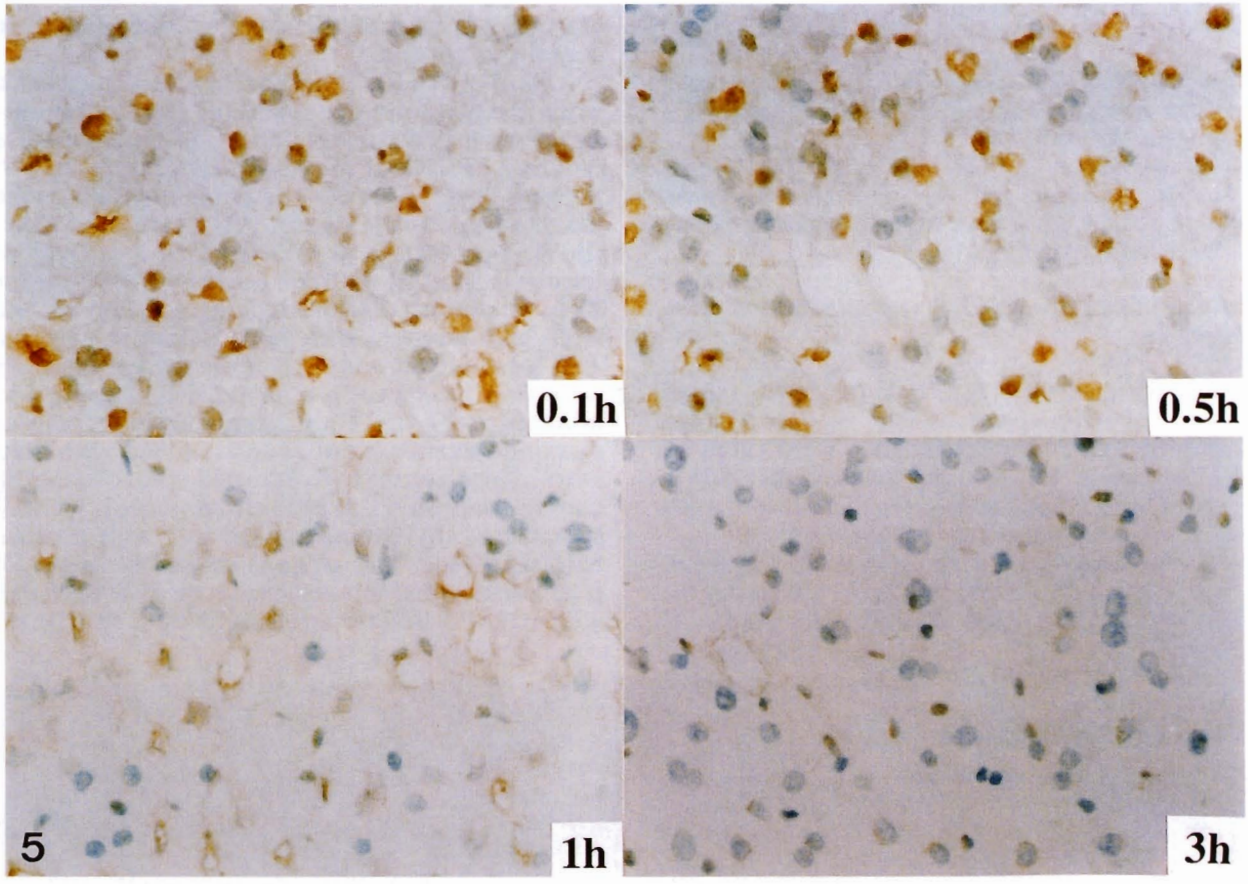

Fig. 5. Tissue sections were stained immunohistochemically to detect DNA fragmentation using the TUNEL method. Brown-colored positive cells appeared immediately after TBI at 2 Gy. x 400

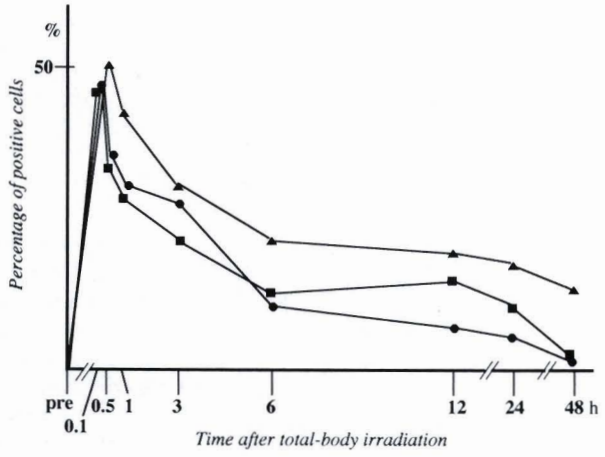

Fig. 6. The time courses of induction of DNA fragmentation detected using an *in situ* apoptosis detection kit in the livers exposed to TBI with 2 (black circle), 7 (black triangle), and 20 Gy (black square).

respectively.

The positive cells of the immunohistochemical-stained sections and apoptotic cells of H-E stained sections were counted by microscopic examination at 320x magnification. Photographs of 5-10 fields per specimen containing approximately 500 cells each were subsequently used to quantify apoptosis.

Results

Survival of irradiated mice

The survival curves of each dose are shown in Figure 1. All mice exposed to 20 Gy γ-ray died within 6 days after irradiation. In the group irradiated at 7 Gy, 50 % of mice died within 11 days, remaining mice survived. None of the mice irradiated at 2 Gy died during the 30 days after treatment. These results indicated that 20 Gy and 7 Gy were corresponding to the doses causing bowel death and bone-marrow death, respectively (Hall, 1988).

Induction of apoptotic cells observed by H-E staining

In the sections stained with H-E, apoptotic cells started to be observed at 0.5 hour after irradiation in the samples treated with 7 Gy or 20 Gy. The percentages of apoptotic cells induced by single doses of either of 7 Gy

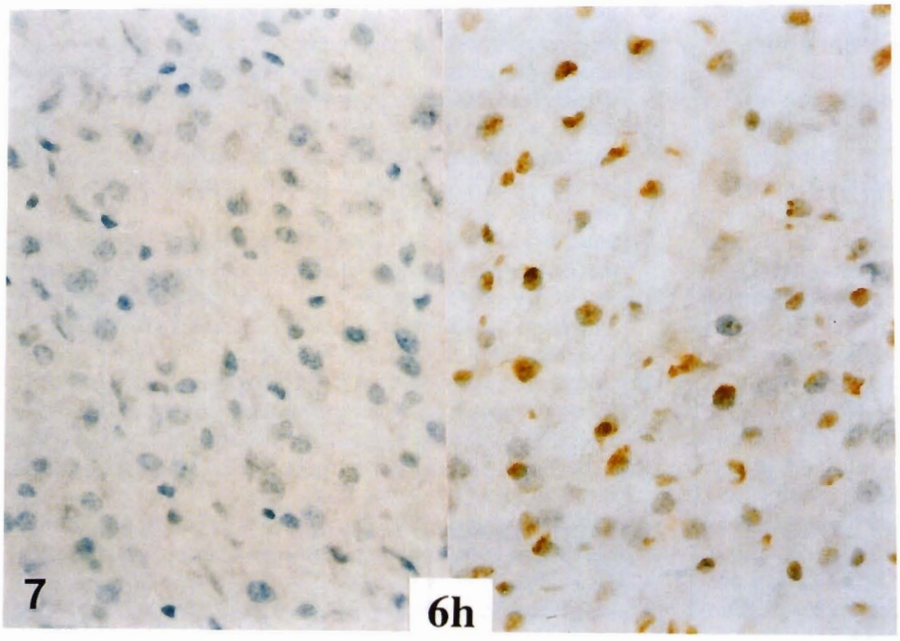

Fig. 7. Effect of CHAPS on the immunohistochemical-stained section to detect DNA fragmentation. At 6 hours after irradiation of 2 Gy, the number of positive cells increases in the section treated with CHAPS (right) compared with untreated sections (left). Positive cells were detected using an *in situ* apoptosis detection kit.

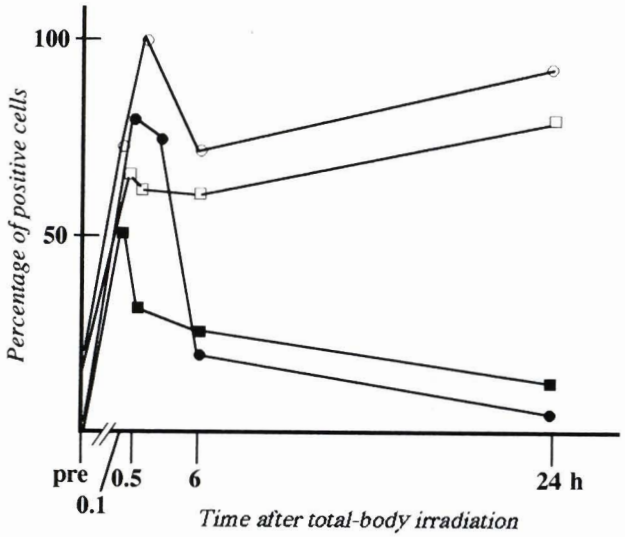

Fig. 8. Effect of CHAPS on the time course of radiation-induced DNA fragmentation. Positive cells in sections of the liver irradiated with 2 Gy are detected using *in situ* apoptosis detection kit (black square, white square) and an *in situ* cell death detection kit (black circle, white cirlce). Time course of positive cells using these staining kits in sections treated with CHAPS are represented as open symbols.

or 20 Gy displayed a curve reaching its highest level 1 hour later (Fig. 2). Apoptotic cells in the samples treated with 2 Gy showed a low number at 0.5 hour. The percentages of apoptotic cells and the length of the period during which the maximum levels of the percentages were exhibited showed a dose-dependent increase.

No necrotic areas were observed in the liver irradiated with either 2 Gy (Fig. 3) or 7 Gy. Necrotic foci appeared 6 hours after the treatment with 20 Gy.

Induction of DNA fragmentation stained in situ

No positive staining for DNA fragmentation was found in the hepatic lobules before TBI (Fig. 4). The positive cells appeared immediately after irradiation (Fig. 5) and they showed no dose dependency in any group (Fig. 6). The percentages peaked immediately after irradiation and declined until they reached a low level. The period exhibiting maximum level of positive cells was shorter and the maximum percentage was higher than corresponding measurements for apoptotic cells in H-E stained sections in both cases at doses of 2 Gy and 7 Gy.

Discussion

Radiation-induced hepatic injury is characterized structurally by progressive fibrous obliteration of central veins (Fajardo and Colby, 1980). In this case report, the veno-occlusive disease involving the central veins in the irradiated area appeared within 90 days of initiation of radiation therapy. Many clinical and experimental reports have been described using radium or low-dose-rate irradiation (Bollinger and Inglis, 1933; Warren and Friedman, 1942; White et al., 1955; Ogata et al., 1963; Lewin and Millis, 1973; Fajardo, 1989).

However, immediate changes induced by high dose-rate irradiation observed by the *in situ* staining method to detect DNA fragmentation have never been reported. We studied TBI-induced DNA fragmentation, detected by the immunohistochemical staining method, and compared it with apoptosis whose biochemical event is

DNA fragmentation prior to visible histological changes, detected by H-E staining (Bursch et al., 1990; Walker et al., 1993; Kerr et al., 1994).

Although apoptosis occurs continually in slowly proliferating cell populations such as the epithelium of the liver (Kerr et al., 1994) in adult mammals, no positive cell was detected in pre-irradiated hepatic lobules (Fig. 4). The results suggest that nuclei in normally proliferative cells are not stained with this method because they have relatively insignificant numbers of DNA 3'-OH ends and are removed from the tissue immediately.

Some murine tumors show a dose dependency for the development of apoptosis *in vivo* when assayed 4 to 6 hours after irradiation (Stephens et al., 1991; Waters, 1992; Akagi et al., 1993; Meyn, 1993, 1994). In the normal liver, apoptotic cells are also maintained at the highest percentage for about 3 hours after irradiation, both in the case of 7 Gy and 20 Gy, and they increased in a dose-dependent manner. In contrast, the highest percentages of positive cells for DNA fragmentation are measurable in samples without a dose dependency. The dose responsiveness of DNA fragmentation appeared to plateau at a dose above 2 Gy in murine liver (Fig. 6). We observed a clear difference between the time course of percentages of apoptotic cells and that of positive cells for DNA fragmentaion in the murine liver, whereas the difference was not observed in other organs including the spleen, kidney, adrenal gland, brain, and small intestine (data not shown). We assume that DNA-binding molecules might obscure detection of DNA fragments with 3'-OH end, causing a discrepancy in the time courses for apoptosis and DNA fragmentation. In order to examine this assumption, we treated the sections of murine liver irradiated at 2 Gy with a detergent.

In the sections treated with CHAPS, a larger number of cells positive for DNA fragmentation were observed than those positive for apoptosis (Fig. 7). At 24 hours after irradiation, when the percentage of positive cells declined to nearly basal levels in the sections without treatment, the percentage of positive cells in the sections treated with CHAPS was still high, over 70% (Fig. 8). About 10% of positive cells were observed in the pre-irradiated samples. In contrast, treatment with CHAPS showed no effect on the number of apoptotic cells detected by H-E staining (data not shown).

This result suggested that the 3'-OH ends of the DNA fragments induced by TBI, like the DNA nicks generated by proliferation, were masked by a detergent-soluble DNA-binding molecule which might be preferentially present in the murine liver.

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