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Evaluation of apoptosis after ionizing radiation in feeding and starving rats

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Abstract. It has been known that γ-irradiation usually induces cell death in regenerating stem cell in normal tissues like skin, intestine and hematopoietic organ. The experiment were carried out to evaluate the early response of radiation injury in radiosensitive and intermediate radiosensitive tissues in feeding and starving rats with the doses of 3.5 and 7.0 Gy. The results of the study showed that the histological phenomenon was apoptosis in the doses of the radiation as the early response of tissue injury. Apoptosis were showed organ-specific and cellular specific responses suggesting that the selection of apoptosis be exactly focused on highly renewal organs and cells. It was interesting that the rats starved for 72 hours prior to irradiation induced less apoptosis in liver than fed rats. As for cellular responses, it appeared that apoptotic cells were mostly distributed in ductal or periportal cells in liver of feeding rats, unlikely in liver of Starving rots which showed no difference in zonal distribution. In salivary gland, apoptotic cells in fed rats were highly induced in intercalating and ductal cell population than in acinar cell population, although unlikely in starved rats. This study showed the value of apoptosis using the detection system of TUNEL for evaluating cellular damage after radiation injury and the diminished effect of starvation on cell damage after ionizing irradiation.

Key words: adult tissues, apoptosis, radiation injury, starvation

Introduction

Ionizing radiation is commonly used in the treatment of tumors but can cause significant damage to surrounding normal tissues.[1] Ionizing radiation has known to induce cell death in the early response to radiosensitive tissues which are susceptible to spontaneous apoptosis in normal adult tissues.[2] As to cellular responses, ionizing radiation has known to induce injury on proliferating cell,[3] participating especially on regenerating stem cells such as intestinal crypt cells,[4] basal cells[5] and hematopoietic stem cells.[6,7] Recently it was reported that the stem-like cells in subependyma which can be a source of oligodendrocytes and astrocytes might be a potential target cell population to irradiation injury.[8] The killing of male germ cells were reported to be closely related to apoptosis after irradiation.[9] The liver has well known to be a radioresistent organ, but the sensitivity to radiation has still been unknown after stem cell activation. Liver stem-like cells are suspected to be progenitor cells which can differentiated into hepatocyte and biliary lineage cells after injury.[10,11,12] Cell kinetics of proliferating stem cell in adult tissues are of considerable importance to radiation effects in tissues,[13,14] since the proliferating stem cells in adult organs might

clonogenically be target cells to irradiation. Also, the early reacting tissues should be in the active cell cycle and rapid cell proliferation and take place in response to tissue injury after irradiation.[15]

Apoptosis is known as a physiologically programmed cell death but it is recognized that cells may die through apoptosis after certain insults. The role of apoptosis in radiation-induced cell death in liver and salivary gland has not been well characterized, especially on stem cell resident in adult organs.

There has been no information that malnourished patients have an increased risk of radiation injury compared to normally nourished individuals. It was reported that oxidant-mediated organ damage may be one cause of increased morbidity and mortality in malnourished patients through low production of glutathione,[21] an antioxidant. To evaluate one possible explanation for this finding, we tried the evaluation of cell death after irradiation in normally fed or starved rats. Although when cells are exposed to γ-irradiation, they are injured primarily by free radicals, in vivo response to radiation injury in tissues should be reevaluated under the condition of dietary restriction or the cellular metabolic activity.

This study was carried out to evaluate the early cellular response of tissue injury and restitution in intestine, liver, salivary gland and lymph node after irradiation with the doses of 3.5 and 7.0Gy, and to compare the effect of starvation to normal feeding in irradiated rats.

Materials and Methods

Animals

Animals tested in the study were male F344 rats weighing 120 to 130g. For time- and dose-depending assay on radiation effect, 22 rats were irradiated on the dose of 3.5Gy and other 22 rats were irradiated on 7.0Gy. Two rats were sacrificed 1/2, 1, 2, 3, 6, 12, 24, 48 and 72 hours in each time point after the irradiation under ether anaesthetic condition. Twelve rats were starved for 72 hours before irradiated with the dose of 7.0Gy to determine the effect of starvation on irradiation. The animals were maintained, three per polycarbonate cage with commercial laboratory chow, under 12 hr/12 hr light/dark cycle-controlled room(21℃±1, 55% humidity).

Irradiation

Rats were given whole-body irradiation with single doses of 3.5 and 7.0 Gy without anesthesia at a dose rate of 100.4 cGy/min, 70 cm in a distance from the source, using 60Co(Theratron 780, AECL, Canada) γ ray. Rats were restrained in an acrylic container of 5x20x20 cm in size.

Histologic examination

After the examination of gross findings, liver, small intestine, submandibular salivary gland and mesenteric lymph node were removed and fixed in 10% buffered formalin and in absolute metharcan. In addition ileum, jejunum and colon were removed for the comparison to the small intestine. After the fixation for 24 hours, tissues were processed routinely, and embedded in low melting point paraffin wax. The sections were stained with hematoxylin and eosin for morphologic examination. The number of positive cells was counted under a light microscopy with a measuring lens(BX50, Olympus, Japan) of x400.

PCNA immunohistochemistry

Tissue sections were deparaffinized in xylene and hydrated in degraded alcohol series, followed by blocking endogenous hydrogen peroxidase with 3% H_2O_2 for 10 minutes. For blocking nonspecific reaction, sections were incubated with normal blocking reagent(DAKO LSAB kit, lot

#00067) for 30 minutes. PC10(DAKO, code #M0879) is a monoclonal antibody against proliferating cell nuclear antigen(PCNA). The tissue sections were incubated with PC10 at 37℃ for 60 minutes. The dilution titers were 1:50 for PC10. Biotinylated mouse immunoglobulin as a secondary antibody was incubated for 10 minutes and peroxidase-conjugated streptavidin was incubated for 10 minutes on tissue sections.

In situ labeling of DNA breaks

Apoptosis(DNA fragmentation) was detected by terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP)-biotin nick end labeling(TUNEL) methods. After were washed in PBS for 20 minutes, deparaffinized sections were digested with proteinase K(20㎎/㎖) for 10 minutes. After removing of endogenous peroxidase, the sections were incubated at 37℃ for 1 hour in a solution containing TdT and digoxigenin-labeled dUTP and deoxyadenosine triphosphate(dATP). The sections were then treated with the peroxidase-labeled anti-digoxigenin antibody for 30 minutes. Diaminobenzidine as a chromogen was developed on the reacting slides. All reagents were purchased from Sigma Chemical Co. (Saint Louis, MO) and Oncor Ltd(Gaithersburg, MD).

Proliferation and apoptosis analysis

The labeling indices for PCNA and apoptotic positive cells were expressed as percentages of the number of labeled nuclei divided by the total number of nuclei in each positive zone. The labeled cells were counted over 10 proliferating zones in a specimen from the highest to the lowest zones. Each calculated value was presented as a mean.

Results

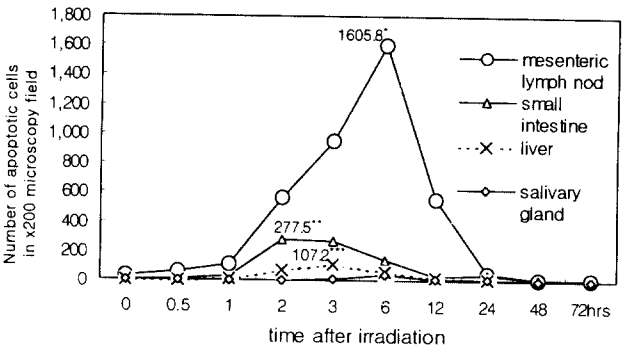

Fig. 1. The number of apoptotic cells at 7 Gy in different tissues of normal feeding rats after irradiation.
Mean number* in mesenteric lymph node at 6 hrs is significantly higher than in small intestine** at 2hrs and in liver*** at 3hrs($p<0.01$).

Time- and tissue-specific induction of apoptosis

Time- and tissue-specific induction of apoptosis were assessed by measuring the number of apoptotic cells in different time and tissues using in situ end labeling method for the assessment of injury of γ-irradiation. Mesenteric lymph node and small intestine were selected as radiosensitive organs, and liver and salivary gland as relatively intermediate radiosensitive organs. The pattern of apoptosis in organs was appeared differently depending on the radiosensitivity of organs and the time sequence. The results showed that responses of apoptosis were organ-specific and cellular specific responses within 24 hours after irradiated with the doses of 3.5 and 7.0Gy in intestine, liver, salivary gland and lymph node in fed rat. Fig. 1 showed the changes of apoptosis induced according to time- and tissue-specific patterns in normal fed rats after the irradiation of 7.0 Gy. The number of apoptotic cells were differently shown according to tissues. The lymph node and small intestine have apoptotic cells more than liver and salivary gland(Fig. 2). The peak number of apoptosis in mesenteric lymph node was significantly higher

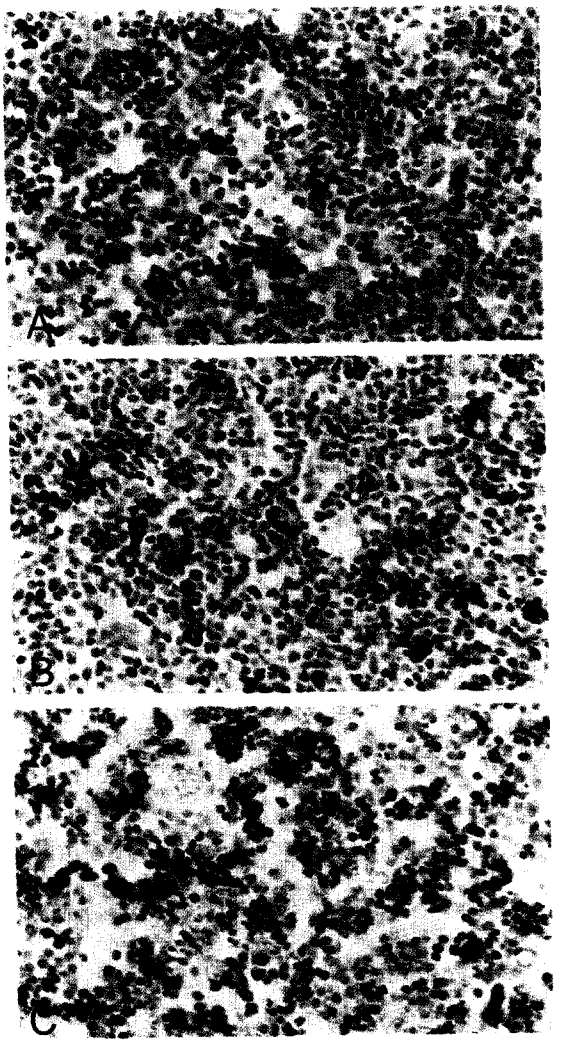

Fig. 2. Mesenteric lymph nodes in male F344 aged 12 weeks old, show apoptotic cells and bodies depending on time sequences. A. at 30 minutes after γ -irradiated with the dose of 7.0 Gy, The grade of apoptosis is similar to normal lymph node. B. at 2 hrs after the irradiation. C. at 6 hrs after the irradiation. Apoptotic cells and bodies are detected by TUNEL method. Counterstained with Auto Hematoxylin(A-C). All is x400.

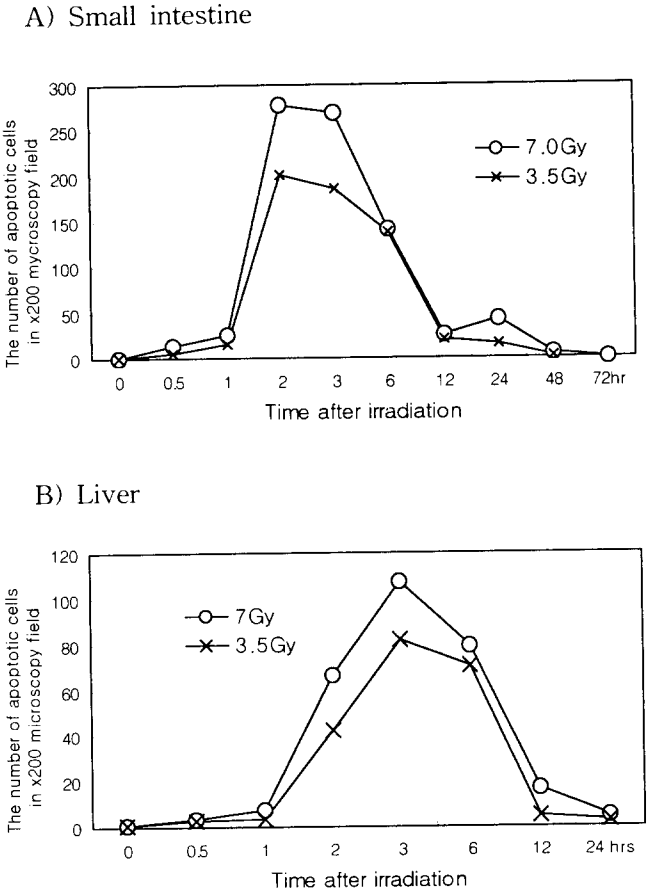

Fig. 3. Apoptosis induction in small intestine (A) and in liver(B) after irradiated with the doses of 3.5Gy and 7.0Gy during feeding. Apoptosis is induced depending on dose sequence in each time point.

than other tissues ($p<0.01$). For the time dependency, in liver and small intestine there were peaks in number of apoptotic cells 2 and 2 to 3 hours, respectively, after the irradiation. In mesenteric lymph node and salivary gland, there is a difference in the time of injury, i.e., 6 hours after the irradiation from those of liver and small intestine. The apoptotic cells in lymph node tissue sections were distributed one-third at 6 hours after irradiated with the dose of 7.0Gy on in situ labeling detection(Fig. 2.C).

Dose-dependent and cellular-specific induction of apoptosis in fed rats

Fig. 3 showed the kinetics of apoptosis to compare the apoptotic induction according to the doses of 3.5Gy and 7.0Gy of γ -irradiation on *in situ* assay in small intestine and in liver during

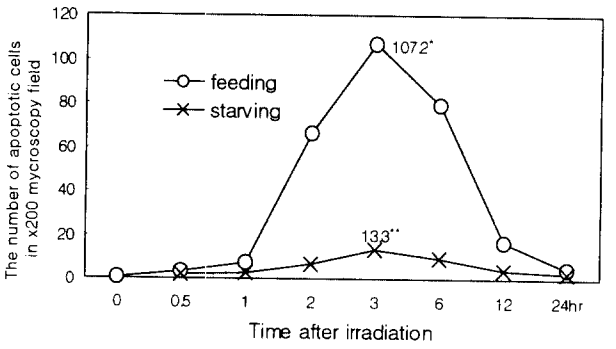

Fig. 4. Apoptosis is very highly induced in liver after irradiated with 7.0Gy when compared of fed rat liver* to starved rat liver**($p<0.01$).

normal diet. The number of apoptotic cells was highly induced with 7.0 Gy, rather than 3.5 Gy in small intestine as well as in liver (at 2 and 3 hrs after irradiation, $p<0.05$). The apoptotic cells in small intestine were mostly counted in crypt. In liver, apoptosis was induced mostly in bile duct cells and hepatocytes in periportal areas including endothelial cells (Fig. 5.B). In salivary gland apoptotic cells in feeding rats were highly induced in intercalating and ductal cell population than in acinar population (Fig. 5.D),

Effect of starvation on apoptosis

For studying the effect of starvation on apoptosis, the responses of apoptosis were examined and compared in starved rats to those in normally fed rats. In small intestine apoptosis was less induced by starvation. However there was no effect on cellular response by starvation. apoptosis was mostly observed in intestinal crypt cells and little produced in villus epithelial cells., liver, salivary gland and lymph node. It was interesting that the rats starved for 72 hours prior to irradiation far less induced apoptosis in liver than feeding rats (at 3 hours after irradiation, $p<0.01$, Fig. 4). The apoptotic response was very high at 3 hours after irradiation and was decreased at 6 and 9 hours(Fig. 6) in liver of starved rats. As for cellular responses, it appeared that apoptotic cells were evenly distributed in zonal assessment (Fig. 5.A). In salivary gland of starved rats, apoptosis was a little induced in ductal and intercalating cell population, showing negative response to fasted rats(Fig. 7). As for repairing response, apoptotic cell death were completely disappeared in lymph node, intestine, liver and salivary gland after 24 hours after the irradiation. Proliferating cell nuclear antigen was highly expressed in liver two to 24 hours after the irradiation.

Discussion

The present study was carried out to evaluate apoptotic responses in different tissues such as small intestine, mesenteric lymph node, salivary gland and liver of normally fed and starved rats after γ-irradiation with the dose of 3.5Gy and 7.0Gy. The results of the study showed that an early response was apoptosis known as a phenomenon of cell death. Apoptotic cell death was confirmed histopathologically and in situ end labeling method for the detection of nuclear fragmentation. With such doses no other histologic finding was seen. It appeared that the frequency of apoptotic cells in feeding and starving rats was well correlated the sensitivity of organs as shown in Stephens' results.[17] The number of apoptotic cells was significantly higher in mesenteric lymph node than small intestine, liver and salivary gland. The induction of apoptosis was maximized in mesenteric lymph node over 60% at 6, in liver at 3, in small intestine at 2-3 hours after irradiation. In normal adult tissues γ-irradiation clonogenically induced regenerating stem cell, i.e. intestinal crypt cells[16] and basal cells in skin,[5] but it is unknown that the target cell of radiation injury can be stem cell in other adult organs than skin, intestine and lymphatic tissues.

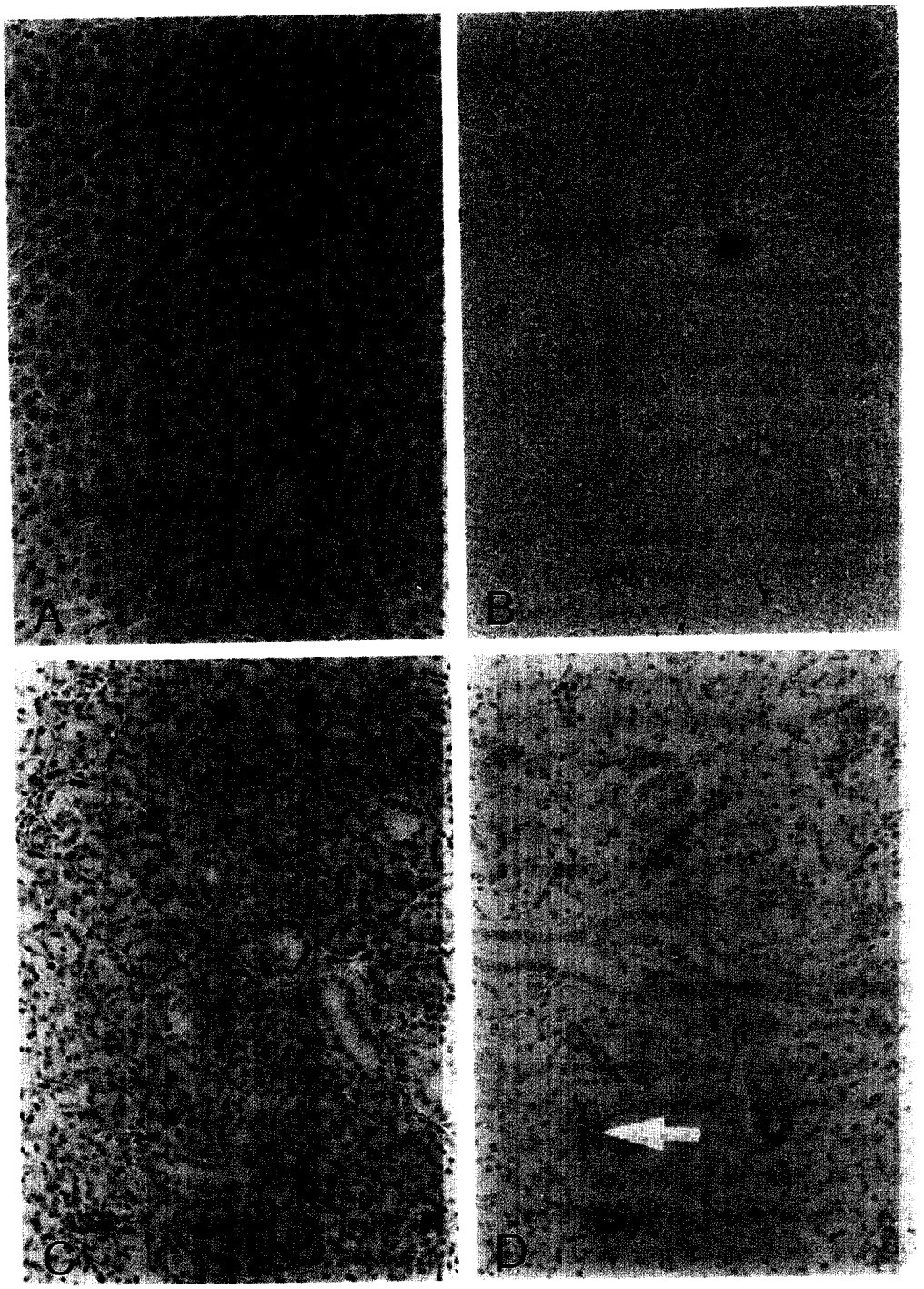

Fig. 5. The comparison of apoptotic induction by 7.0Gy of γ-irradiation during starvation for 72 hrs to normally feeding in livers(A and B) and salivary glands(C and D) of F344 rats. The apoptotic cells at 3 hrs after the irradiation are mostly distributed in periportal area in livers of normally fed rats(B), on the contrary, not in liver of starved rats. Similarly apoptosis is induced in intercalated ductular and ductal cells in salivary gland during feeding(D), not in starved rats(C). Apoptosis detection by TUNEL, Counterstained with Auto hematoxylin(A and C) and ethyl green(B and D). All is x200.

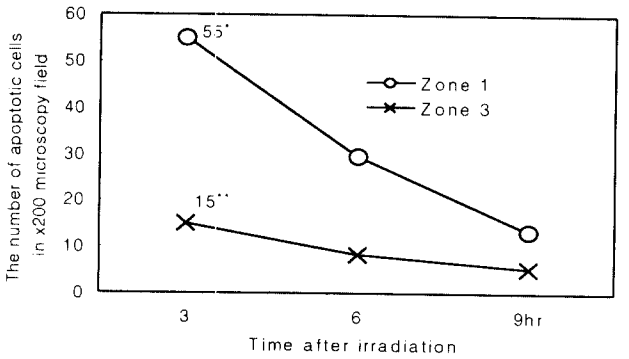

Fig. 6. Apoptosis induction is significantly different between zone1* and zone3**($p<0.05$) in liver irradiated during feeding at 3.5Gy

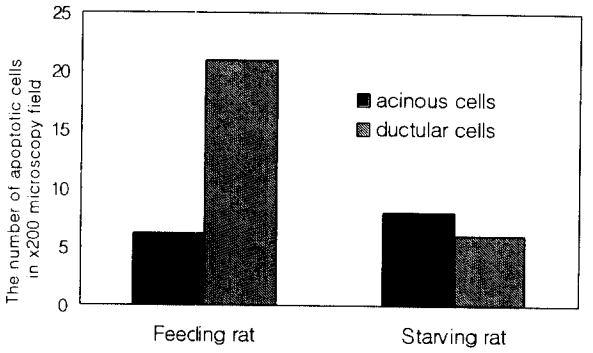

Fig. 7. Difference of apoptosis induction during feeding and starvation between acinous and ductular cell population of serous salivary gland 6hr after irradiated at 3.5Gy

Fig. 8. Apoptosis detection by TUNEL method. Apoptotic cells are clonogenically induced in regenerating crypt cells in intestine(A, at 2 hrs) and periductal cells in liver(B, at 3hrs) after γ -irradiation of 7.0 Gy of the rats. Conterstained with ethyl green. A. x100; B. x400.

That's why the being of stem cells in liver and salivary gland has still not be clearly defined. The apoptotic cells in the study were distributed mostly in periportal cells and duct cells in liver(Fig. 8.A) and intercalated and ductular cells in salivary gland (Fig. 8.B), suggesting that the apoptotic cells might be clonogenically proliferating stem-like cells. Because it is natural to think that the stem cell in adult organs is defined to be normally regenerative and regenerating stem cells are clonogenically susceptible to ionizing irradiation at cellular replacement(cellular turning over).

There was an interesting difference in extent of induction of apoptosis and cellular response between starved and fed rats. Apoptosis was more induced in feeding rat compared to starved ones, and the number of apoptosis was very high in periportal areas and low in pericentral area in feeding liver. On the contrary, there was little difference in zonal distribution of apoptotic cells in starved rats. The findings suggest that hepatocytes around periportal areas of feeding rats are different in radiosensitivity from those in starved rats. There was a similar finding in

small intestine between fed and starved rats, showing histopathologically apoptosis mostly in crypt cell. The number of apoptotic cells in crypt of small intestine of feeding rats was as much higher as 60-70% than starving rats. Radiation -induced apoptosis was reported to be targeted in cells with active cell cycle during regeneration in normal and abnormal condition of tissues.[2]

Apoptosis with the cellular activity as well as metabolic activity might be high in liver of feeding rats, suggesting that high metabolic activity in periportal areas during feeding, when compared to starvation, has the different effect on radiosensitivity in different cells. Therefore, high frequency in apoptosis in tissues of feeding rats means that cellular and metabolic activity in periportal areas should be liable to radiation injury as reported previously.[18] Periportal to perivenous gradients of oxygen and zonal differences in the hepatic innervation,[19,20] seem to be partly responsible for apoptosis expression. It is well established that cellular injury by low dose of irradiation is due to the production of free radicals. Although it was not assayed the extent of free radical formation and the metabolic activity, it could be suspected that the decreasing metabolic activity by starvation that might produce less free radicals resulted in the low level of apoptosis in liver and small intestine of starved rats. However this interpretation is so different from Robinson's results[21] showing that starvation can enhance hepatic free radical release following endotoxemia stress due to the depression of liver glutathione, an antioxidant against free radicals. In regard to this point, antioxidants and/or the scavengers of free radicals should be tested for the restitution of injured tissue.

This study showed the value of apoptosis detection system for evaluating cellular damage against radiation injury. Because apoptosis was regularly induced depending on tissue-specific pattern, dose and time sequence as well as cellular activity. Furthermore in vivo model in the study will be helped in the further study to elucidate the relationship between radiation injury and starvation and/or malnutrition.

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