Measurement of Apoptotic Fragments in Small Intestine following γ-Ray Irradiation in Mice

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요 약

방사선의 피폭선량을 예측하기 위한 짧은 시간내 검색이 가능한 민감성 지표를 개발하기 위해 소장 crypt cell에서의 유도된 apoptosis의 빈도에 대해 알아보았다. ^{60}Co γ-ray를 whole body에 0.25의 저선량에서 8Gy의 고선량까지 조사하여 소장의 음와세포에서 apoptic cell의 수적 변화를 관찰한 바 방사선 조사 후 시간의 경과와 선량의 증가에 의존적이었다. 소장 음와세포에서의 apoptotic cell의 수는 방사선 조사 후 4-6시간에 최고치를 나타내었고, 24-48시간 후에는 점차적으로 감소하여 72시간 후에는 현저히 감소되었다. 또한 방사선의 선량의 증가에 따라 소장 음와세포에서의 apoptotic cell의 수가 현저히 증가하였다. 방사선 조사에 의한 음와세에서의 apoptosis의 발생빈도에 대한 선량 반응 곡선은 linear-quadratic model로 분석되었고, 그 식은 $y = 0.18 + (5.125\pm0.601)D + (-2.652\pm0.7000)D^2$ (r^2=0.970) 이었다 (D = 조사선량 또는 피폭 선량).

이상의 결과로부터 생체내에서 방사선에 의해 소장 crypt cell에서 유도된 apoptosis의 빈도는 방사선 조사 선량간에 밀접한 관계식이 성립됨을 알 수 있었다. 따라서, 소장 음와세포에서의 apoptotic fragment assay는 간편하고 빠르며 재현성이 있는 지표로서 방사선 피폭으로 발생된 세포 손상의 생물학적 영향 평가, 방사선 방호제의 민감도 검사, 방사선 동위원소의 체내 오염에 대한 체내 피폭선량 예측 지표 및 방사선 민감 장기의 손상 정도의 확인에 이용 가능할 것으로 사료된다.

Abstract

We have examined induction of apoptosis by low dose irradiation for the possibility as a short-term biological dosimeter in small intestinal crypt cells of the Balb/c mice. Measurements were performed on apoptotic fragments frequencies per 500 crypt cells for section after whole body ^{60}Co γ-ray irradiation in the range of 0.25 to 8 Gy. We observed a significantly effective induction of apoptosis at 4 and 6hours after irradiation. There was a significant dose-response relationship between the frequency of induced apoptotic fragments and dose of γ-rays. The dose-response curves were analyzed with linear-quadratic models: frequencies per crypt cell were $0.18 + (5.125\pm0.601)D + (-2.652\pm0.7000)D^2$ (r^2=0.970) after the γ-ray irradiation(D is the irradiation dose in Gy). These results indicate that the detection of apoptotic fragments showed the strong possibility as a short-term accident biological dosimeter. In addition, it can be a reliable tool for dose response evaluation of low dose γ-ray irradiation.

Key words; ^{60}Co γ-ray, intestinal crypt cell, apoptotic fragment assay, biological dosimeter

Introduction

Ionizing radiation-induced cell death has been

studied extensively in a wide variety of cell types and cell lines. Both apoptosis and classical necrosis, genetically, biochemically and morphologically fundamental different types of cell death, have been recognized, not only depending on the cell type but also on the radiation dose(Hertveldt et al., 1997). Apoptosis is a physiological mode of cell death requiring active cellular processes(Arends et al., 1990). This process is distinct from general tissue necrosis. Morphologically, its gross features, identified by EM, include nuclear chromatin condensation, compactness of cytoplasmatic or organelles, and the appearance of pedunculated protuberances on the cell surface(Kerr et al., 1987). Light microscopy of apoptosis shows that intensely basophilic, corresponds in distribution with the condensed chromatin observed by electron microscopy. It seems that chromatin cleavage is the most characteristic biochemical feature of the process(Tian et al., 1991). In well-established model systems, large frgments of 300kb and 50kb are first produced by endonucleolytic degradation of higher-order chromatin structural organization. Thus, the appearance of the ladder of nucleosomal DNA fragments in agarose gels became the hallmark of apoptosis. The DNA fragmentation is responsible for both staining with TUNEL assay and DNA laddering, The labeling target of the TUNEL assay is the multitude of new 3′-OH DNA ends generated by DNA fragmentation and typically localized in morphologically identifiable nuclei and apoptotic bodies. In contrast, normal or proliferative nuclei which have relatively insignificant numbers of DNA 3′-OH ends, do not stain. This mixed molecular biological-histochemical system allows for sensitive and specific staining of the very high concentrations of 3′-OH ends that are localized in apoptotic bodies.

In biological dosimetry, it is a difficult task to confidently measure the biological damage and subsequent risk associated with radiation exposure. Chromosome aberration and micronucleus formation are classical biological endpoints that have been used to assess radiation damage to a cell(Muller et al., 1991). Ionizing radiation damages DNA and consequently causes the formation of chromosome aberrations. The frequency of aberrations is therefore a good indication of the effect of the radiation exposure (Muller et al., 1991). Analysis of chromosome aberrations, however is time consuming and requires skilled technical support. Alternatively, the micronucleus assay is an indirect measure of DNA damage that is somewhat faster and requires less technical expertise(Hall and Wells, 1988 ; Fenech, 1993). There is a controversy regarding the potency of micronucleus assay as an indicator of radiosensitivity, indicating that unknown factors may influence the assay.

At low radiation doses, this variability, along with other factors, makes biological dose estimation difficult. For the purpose of biological dosimetry, an endpoint that facilitates post-exposure calibration would provide greater flexibility and improved sensitivity. In this work we describe the development of the method for an *in situ* labeling of DNA breaks in nuclei, in this sections processed through the routine procedure of histopathology, and its utilization for study of tissue dynamics.

We propose that apoptosis of intestinal crypt cells may be a good parameter that would be useful for biological dosimetry and for identifying radiosensitivity. In addition, we take the opportunity to give some considerations on the kinetics of apoptosis in low dose-rate exposures.

Materials and Methods

Animals

Balb/c mouse 7 to 8 weeks old and weighing 25-30g were used in all experiments. Mice were housed five to a cage and allowed NIH-07 diet and water *ad libitum*. The conventional animal facility was the Laboratory of Experimental Animals Care and Management, Korea Cancer Center Hospital. The animals were housed, five to a cage, in conventional animal facilities with NIH-07 diet and water *ad libitum* under constant temperature (23℃) and with a 12h light and dark illumination cycle.

Irradiation

All animals were irradiated with a source ^{60}Co irradiator(Theratron-780 teletherapy unit). Briefly, the mice were situated in close-fitting Perspex box(22x 11x 4cm) and received whole-body irradiation doses of 0 to 1.0Gy with a dose rate of 98.2cGy/min in the box. The treatment field was 30mm in diameter. All irradiations were performed in air at room temperature.

Assay of apoptotic fragmentation

The relationship between the increase of dose and the number of apoptotic fragments has been investigated. We represent the number of apoptotic fragments in the small intestinal crypt cells per section. Three different technique that detect apoptosis at different stage of process were used. Slide were stained with H&E.

Also, these were stained 4, 6-diamino-2 phenylindole(DAPI). These stained nuclei and apoptotic nuclei were scored using light and epifluorescent microscope. The second technique used was the TdT-mediated dUTP biotin nick end labeling (TUNEL) assay which measured the specific binding of therminal deoxynucleotidyl transferase(TdT) to 3'-OH ends of DNA, ensuing a synthesis of polydeoxynucleotide polymer. Briefly, apoptotic nuclei have fragmented DNA that was labeled with digoxigenin-nucleotides by TdT reaction. The incorporated digoxigenin nucleotides were detected with fluorescen labeled anti-digoxigenin antibodies(Gavrieli et al., 1992). The TUNEL assay was performed using a commercial apoptosis detection kit(Oncor Chemical Co). Values for the TUNEL assay were scored the number of positive apoptotic cells in the crypt cells.

Kinetics of radiation-induced apoptosis

Since apoptosis is known to be a time-dependent event, animals were exposed to 0.75Gy of γ-ray maintained up to 72h postirradiation. All animals were autopsied at various times and assayed for appearance of apoptotic cell using the H&E stain and TUNEL assay.

Statistic alanalysis

For statistical analysis of measurements from each sample, the significance was assessed by Graph PAD In Plot computer program(GPIP, Graph PAD Software Inc., San Diego) and EXCEL software program. Standard error bars are shown for each data point except where the error is equal to or less than the symbol size.

Results

Kinetics of radiation-induced apoptosis

To find dose response relationship after γ rays irradiation, the number of apoptotic fragments were counted in crypt cells of mice by light microscope(LM): the number of apoptotic crypt cells was obtained by subtraction of the number

of cells scored as apoptotic cells in the control samples from the total number of those cells in the irradiated samples. The morphological findings of the irradiated groups were typical apoptotic cells in intestinal crypt cells as shown in Fig. 1. The apoptosis in crypt cells was maximal at 4 and 6hours after irradiation, showed a gradual decline at 24 and 48hours, and was almost absent by 72hours(Fig. 2A). After irradiation, the number of apoptotic cells increased sharply with increasing dose of γ rays(Fig. 2B). The highest frequency of apoptosis in crypt cells was seen at 1Gy and then declined gradually beyond the dose of 2Gy with high levels of damage. There was a significant correlation between the frequency of apoptosis in crypt cells and the dose. However, the spontaneous apoptotic frequency in crypt cells of the irradiated groups showed no significant difference between individuals. These results indicate that the apoptosis in crypt cells shows a time and dose dependent increase.

Dose response relationship of radiation induced apoptosis

As shown in Fig 1, the morphology of apoptotic cells displayed evidences different from that of necrotic cells. The data obtained in dose response study are presented in Fig. 3. The spontaneous apoptotic frequency in crypt cell of unirradiated animals was not significantly different from individuals. The baseline number of apoptotic cells per crypt cell in unirradiated animal was low, being 0.18±0.0282(Mean±SE, Fig 2). There was a significant relationship between the frequency of induced apoptotic fragments and dose of γ ray. The dose response curves were analyzed with linear quadratic model: frequencies per crypt cell were $y=0.18+(5.125\pm0.601)D+(2.652\pm0.7000)D^2$ ($r^2=0.970$) after the γ-ray irradiation(Y is the number of apoptotic cells/500 crypt cells and D is the irradiation dose in Gy).

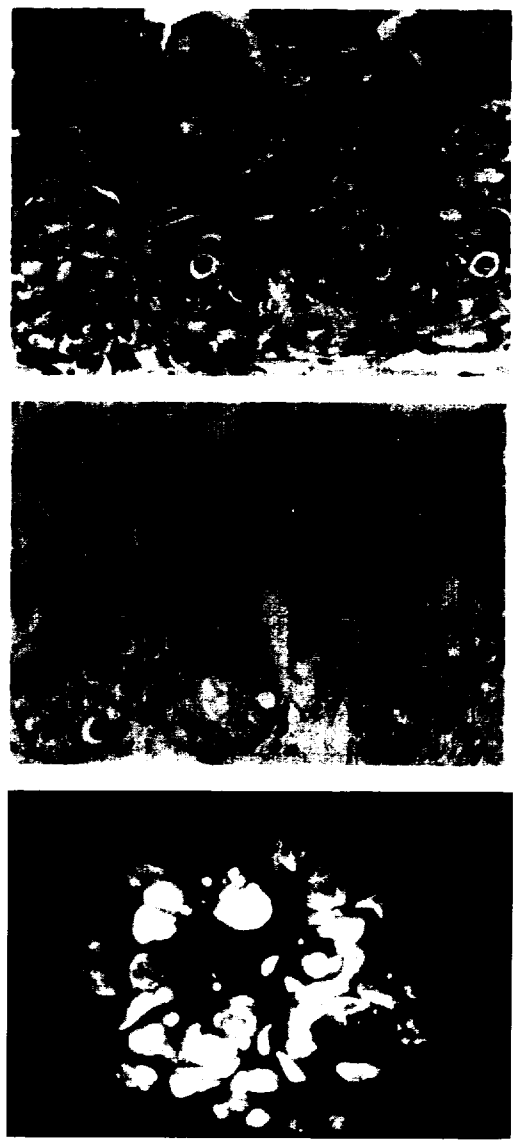
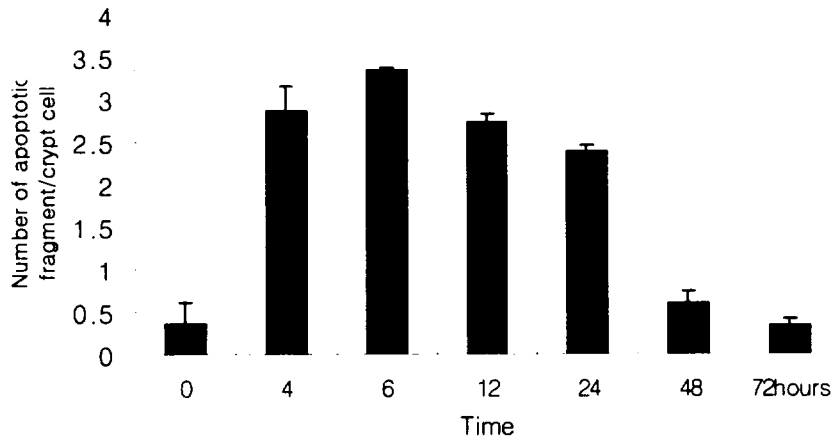


Fig. 1. Morphological classification of typical apoptotic fragments in the crypt cells. These cells are scored as apoptotic fragment(arrows). Upper plate presents typical apoptotic fragments stained with H&E(×132). The cells show chromatin condensation into crescentric caps at the nuclear periphery, nuclear disintegration and shrinkage of cell volume. Middle plate shows poptotic fragments with TUNEL positive nuclei by TUNEL assay(×132). Lower plate shows apoptotic nuclei stained with DAPI(×264).

A



B

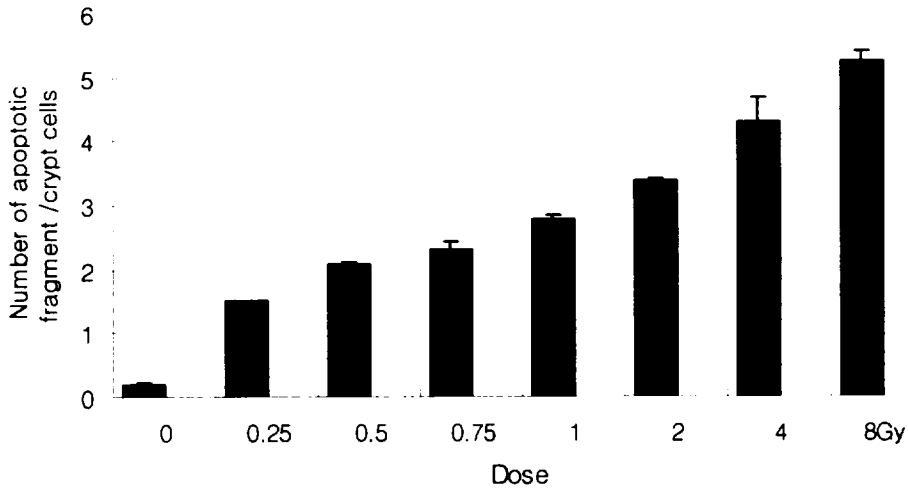


Fig. 2. Time(A)- and dose(B)-dependent apoptosis induction in mice crypt cells after ^{60}Co γ-rays irradiation(N=5 at each tine point). (A) Animals were exposed to 0.75Gy of γ-rays maintained up to 72h post-irradiation. (B) Animals were sacrificed 6hours after irradiation, having reached a maximum yield of apoptotic fragments. The error bars represent the standard deviations.

Discussion

Apoptosis is basically characterized by cellular shrinkage, marked condensation and margination of chromatin, nuclear and cellular fragmentation with well preserved cell organelles. At an early stage, basophilic masses are found around the margins of nuclei in most cell types. Later in the process, the nuclear fragments present in the apoptotic bodies appear as descret basophilic masses(Arends et al., 1990). While large apoptotic bodies can be readily identified in tissues as spherical or roughly ovoid acidophilic globules, irrespective

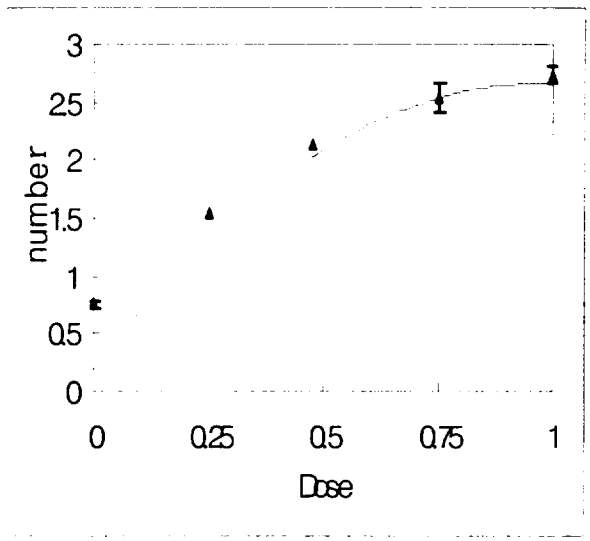


Fig. 3. The dose-response relationship of apoptotic fragment induction in intestinal crypt cells 6hours after irradiation with ^{60}Co γ-rays. The error bars represent the standard deviations. ▲ ^{60}Co γ-rays.

of the presence within them of nuclear component. In addition to, the bodies occur both singly and in small clusters and are sometimes surrounded by clear halos(Kerr et al., 1987). The visualization of focal *in situ* staining inside intact apoptotic nuclei and apoptotic bodies exactly correlates the more typical biochemical and morphological characteristics of apoptosis. Consequently, the apoptosis is generally described as a rapidly occuring process(Rosl, 1992). The time required for apoptotic cell to fragment its DNA varies depending on the organism, cell type, and the type of including signal(Kerr, 1994). In this study, the earlist changes are the occurrence of recognizable apoptotic cells in crypt cells 4h after irradiation. The apoptotic process was decreased within a period of about 48h. The decrease in number of apoptotic fragments result from engulfing and digesting apoptotic bodies by neighbouring healthy cells. Therefore, it is important to obtain sample quickly follwing an external irradiation.

Quantitative measurement of biological responses to ionizing radiation is clearly of the utmost importance in regard to the radiation protection and risk assessment in radiation exposures. The relationship is linear quadratic relationship usually observed for chromosome aberrations(Mitchell, 1987). Since there is a growing need for a simple and reproducible biological dosimeter to use following accidental exposure to various types of radiation, the usefulness of the apoptotic fragment assay is examined the radiation response of small intestine. The characteristics of the dose-response relationship obtained with the detection of apoptotic fragments in the crypt cell after γ-ray irradiation. This change is easy quantifiable and dose dependent. An analysis of mammalian cell radiation-dose survival curves, based on the linear-quadratic formalism, is shown to yield insights in the various component of damage that contribute to cell reproductive death. On the other hand, the detection of apoptotic fragments will require further investigation because of the acquirement of intestinal crypt cell sample.

In conclusion, the stem cell of small intestine is very sensitive to immediate radiation damage. These results indicate that the detection of apoptotic fragments showed the strong possibility as a short-term accident biological dosimeter. In addition, it can be a reliable tool for dose response evaluation of low dose γ-ray irradiation.

Acknowledgements

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