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Mouse model system based on apoptosis induction to crypt cells after exposure to ionizing radiation

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Abstract : To evaluate if the apoptotic fragment assay could be used to estimate the dose prediction after radiation exposure, we examined apoptotic mouse crypt cells per 1,000 cells after whole body ^{60}Co γ -rays and 50MeV ($p \rightarrow Be^{+}$) cyclotron fast neutron irradiation in the range of 0.25 to 1 Gy, respectively.

The incidence of apoptotic cell death rose steeply at very low doses up to 1 Gy, and radiation at all doses trigger rapid changes in crypt cells in stem cell region. These data suggest that apoptosis may play an important role in homeostasis of damaged radiosensitive target organ by removing damaged cells.

The curve of dose-effect relationship for the data of apoptotic fragments was obtained by the linear-quadratic model $y = 0.18 + (5.125 \pm 0.601)D + (-2.652 \pm 0.7000)D^2$ (r^2=0.970) after γ -rays irradiation, while $y = 0.18 + (9.728 \pm 0.887)D + (-4.727 \pm 1.033)D^2$ (r^2=0.984) after neutrons in mice. The dose-response curves were linear-quadratic, and a significant dose-response relationship was found between the frequency of apoptotic cell and dose.

These data show a trend towards increase of the numbers of apoptotic crypt cells with increasing dose. Both the time course and the radiation dose-response curve for high and low linear energy transfer (LET) radiation modalities were similar. The relative biological effectiveness (RBE) value for crypt cells was 2.072.

In addition, there were significant peaks on apoptosis induction at 4 and 6h after irradiation, and the morphological findings of the irradiated groups were typical apoptotic fragments in crypt cells that were hardly observed in the control group.

Thus, apoptosis in crypt cells could be a useful *in vivo* model for studying radio-protective drug sensitivity or screening test, microdosimetric indicator and radiation-induced target organ injury. Since the apoptotic fragment assay is simple, rapid and reproducible in the range of 0.25 to 1 Gy, it will also be a good tool for evaluating the dose response of radiation-induced organ damage *in vivo* and provide a potentially valuable biodosimetry for the early dose prediction after accidental exposure.

Keyword : Intestinal crypt cell; apoptotic fragment assay; mouse model; low dose; early dose prediction; radiation; biodosimetry

Introduction

Radiation exposure is associated with an increased risk of developing myeloid and lymphoid cells malignancies[1]; thus dose estimates and biomonitering are important components of risk assessment. The mechanism underlying apoptosis after radiation exposure are poorly understood. The simplistic paradigm is that radiation induces DNA damage, genetically damaged cells expand either prior to after acquisition of additional aberrations, and, once sufficient genetic hits have accumulated, the cells become transformed. One means of assessing the risk of genotoxins to humans is to develop

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nonhuman biological models in which the dose, route of exposure, cell type, and endpoint examined are matched as closely as possible to those used for screening humans. There is only a limited amount of data comparing the induction of genetic damage by ionizing radiations between human and nonhuman mammalian species, in which morphological changes was used to ensure that only radiation-induced apoptotic crypt cells were scored. Information on the absorbed dose and its distribution is of great important for an early estimation of irradiation consequences, but there hasn't been effective biological endpoint to assess the early dose prediction after internal contamination or external irradiation, especially in low dose exposure. Therefore, the accurate dose-response by simple and informative *in vivo* model system would be of great value in evaluating genetic risk following accidental exposure to radiation: Early dose prediction by the calculated *in vivo* model system will help researchers to estimate the biological effect by comparing the observed yields of radiation-induced apoptotic cells to a standard baseline of spontaneous apoptotic cells.

Apoptosis is a physiological mode of cell death requiring active cellular processes. It can't occur only as a response to internal as well as external stimuli, but also accidental cell death like necrosis which cannot maintain its intracellular homeostasis by severe external damage[2,3]. Radiation-induced apoptosis in thymocytes is characterized by various morphological, biochemical and genetic indicators[4,5]. Its occurrence is dependent on cell type and radiation dose.

With respect to cytogenetic damage, it is well established that high LET radiation is more effective than low LET radiation[6,7]. At low dose range, therefore, it is important to find an endpoint that facilitates post-exposure calibration with greater flexibility, improved sensitivity and retrospective. Differences in relative biological effectiveness (RBE) found between high and low LET radiations are caused by the degree of initial DNA damage and the extent to which they are repaired or misrepaired[6,8]. On the other hand, no differences in RBE values for neutrons versus X-rays were found in thymocytes[4], but there did not consider apoptosis as a mechanism for the interphase cell death. Thymocytes and lymphocytes are very sensitive to immediate radiation damage[9,10]. They undergo early interphase cell death *in vitro* and *in vivo* by apoptosis.

Consequently, to develop *in vivo* model system of a simple cytogenetic technique, we extensively studied ionizing radiation-induced cell damage in a wide variety of cell types and cell lines using morphological, biochemical and genetic markers, and found a possibility of the biodosimetry for radiation exposure using apoptosis in mouse hair follicles[11,12]. Since the stem cells with high radiation sensitivity in the crypt are found at the fourth or fifth position from the base of the crypt[13-17], the dose estimation of crypt cells with high proliferation and radiation susceptibility to apoptosis in all radiation qualities will be an useful model to estimate the absorbed dose in target organs.

In the present study, the experiment was designed to investigate the effectiveness of fast neutrons versus γ-rays with respect to apoptosis induction in mouse crypt cells and to examine whether the apoptotic fragment assay could be an indicator of biological dosimetry for the radiosensitive target organ damage.

Materials and Methods

Experimental Animals

One hundred of Balb/c mice (Laboratory of Experimental Animal, Korea Cancer Center Hospital, KCCH) (7 to 8 weeks old, and 25-30 g) were used in all experiments. The animals were housed in temperature- and humidity- controlled conditions and allowed NIH-07 diet and water ad libitum, and were kept on a high 12-h light cycles. The semi-barrier animal facilities were established in the Laboratory of Experimental Animals, KCCH. This experiment was approved ethically by the rule of institute authorities. Five control and five irradiated animals were used for each experimental group.

Preparation of Intestinal Crypts detection

Animals were anesthetized by an intraperitoneal injection of sodium pentobarbital (1 mg/kg body weight) immediately prior to irradiation. The animals were killed by cervical dislocation, and the jejunum was removed, flushed with normal saline, and fixed with Bouin's solution. The tissues were embedded in paraffin and cut at 3-5 μm thickness.

Irradiation

All animals were irradiated by γ-rays with a source of ^{60}Co irradiator (Theratron-780 teletherapy unit) or fast neutrons generated by cyclotron (MC-50, Scanditronix). Briefly, the mice were placed in closed-fitting Perspex boxes (22x 11x 4 cm) and was irradiated whole-body with γ-rays or fast neutrons doses of 0 to 1.0 Gy with a dose rate of 98.2 cGy/min and

30 cGy/min, respectively. The treatment field was 30 mm in diameter, and irradiation was performed in air at room temperature.

Detection of Apoptosis

Crypts were cut longitudinally and most of the lumen was selected for analysis. Employing distinctive morphological feature of apoptosis, as described by Kerr et al, to recognize apoptotic cells, the incidence of apoptotic cells was quantified by counting the number of apoptotic cell in each crypt, and any doubtful cells were disregarded.

Three different techniques were used to detect apoptosis; Hematoxylin and eosin (H&E), 4, 6-diamino-2-phenylindole (DAPI), and TdT-mediated dUTP-biotin nick end labeling (TUNEL) assay (Oncor Chemical Co.). The stained apoptotic nuclei were scored using light and fluorescent microscope.

Kinetics of radiation-induced apoptosis

Since apoptosis is known to be a time-dependent event, animals were exposed to 0.75 Gy of γ-rays and were maintained up to 72h post-irradiation. All animals were autopsied 0, 4, 6, 12, 24, 48 and 72 h and assayed for the appearance of apoptotic cell

Statistical Analysis

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

Induction kinetics of apoptosis in the mouse crypt cells after radiation exposure

To find dose-response relationship after γ-rays and fast neutrons, the numbers of apoptotic fragments were counted in crypt cells of mice by light microscope (LM), respectively: The number of radiation induced 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 fragments in crypt cells, as shown in Fig 1.

The average numbers of apoptotic crypt cells induced by γ -rays and fast neutrons, obtained by pooling the LM data of the 20 mice, are presented as a function of radiation dose and the error bars represent standard deviations within the studied population of experimental animals. There was a significant peak of apoptosis induction at 4 and 6 h after irradiation with fast neutrons or γ-rays (Fig 2, 3). The number of apoptotic cells increased sharply with both times after irradiation and sizes of dose. The dose-response curves presented in Fig 3 indicate that the apoptotic process in high LET radiation was faster than that in low LET radiation. However, the spontaneous apoptotic frequency in crypt cell of the unirradiated groups showed no significant difference between individuals. The baseline number of apoptotic cells per crypt cell in unirradiated animal was low, being 0.18 ± 0.0282 (Fig 2, 3).

Dose-response relationship

To evaluate the dose-response curves, the number of apoptotic cells per crypt was examined at the different doses, and the dose-response curve of apoptotic crypt cells was obtained by fitting the linear-quadratic model $y=a+bD+cD^2$, where y is the yield of apoptotic crypt cell, a is the spontaneous yield, b is the coefficient of the one-track component, c is the coefficient of the two-track component, and D is the dose in Gy. When plotting on a linear scale against radiation dose, the line of the best fit was $y=0.18+(5.125\pm 0.601)D+(-2.652\pm 0.7000)D^2$ (r^2=0.970) after γ-rays, while $y=0.18+(9.728\pm 0.887)D+(-4.727\pm 1.033)D^2$ (r^2=0.984) after neutrons (Fig 3). There was a significant correlation between the frequency of apoptotic crypt cell and dose. The dose-response curves were linear-quadratic. These data show trends towards increasing apoptotic crypt cell numbers with increasing dose, a plateau being above some higher doses was observed.

Relative Biological Effectiveness (RBE)

To estimate effectiveness of apoptosis induction by the two radiation qualities, the RBE values, as defined by the ratio of γ-ray dose taken as reference radiation quality to the neutron dose required to obtain equal biological effects, was used. As shown in Fig 3 and Table 1, the mean RBE value in mice was 2.072, showing significant difference in the number of apoptotic cells between fast neutrons and γ-rays: The results obtained with three different detection methods of apoptosis were very close.

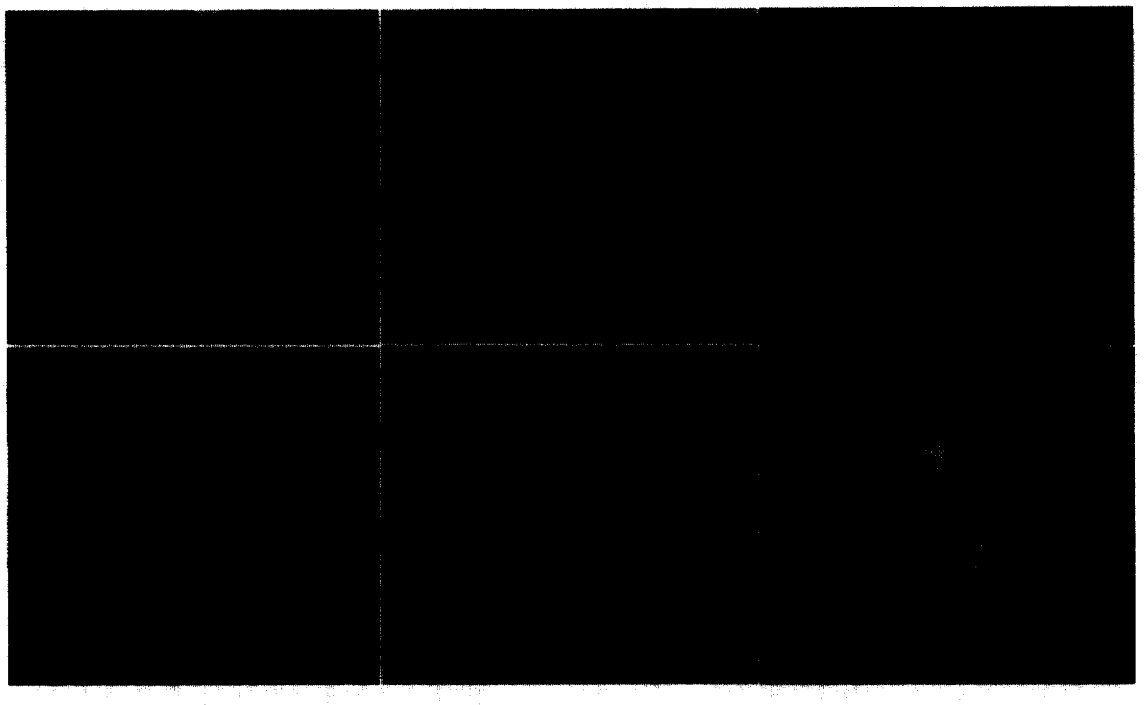

Fig 1. Micrographs of typical apoptotic fragments in intestinal crypt cells with H&E stain(A,B), TUNEL assay(C,D), and DAPI stain(E,F) after irradiation with ^{60}Co γ -ray : apoptotic cells(arrow heads)

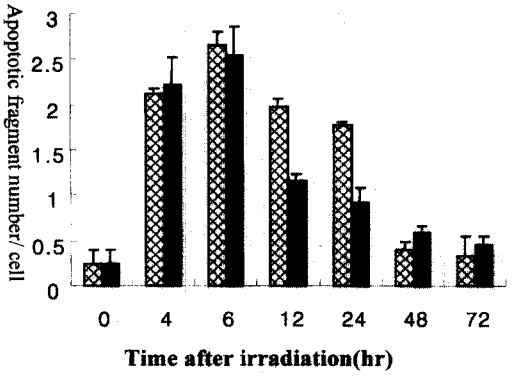

Fig 2. Comparative biological effects of ^{60}Co γ -ray( ) and neutron(●) for specific times as revealed from studies in mouse crypt cells exposed *in vitro*

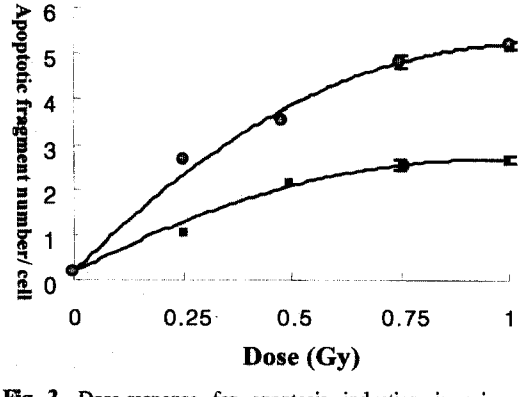

Fig 3. Dose-response for apoptosis induction in mice crypt cells 6h after irradiation with ^{60}Co γ -ray(■) or fast neutron (●). Each point represents the mean ± SD Obtained by pooling the LM scoring data of 20 mice. The error bars represent the standard deviation within the experimental population.

Table 1. RBE of neutrons compared with ^{60}Co γ -rays for inducing apoptotic fragments in mice crypt cells exposed *in vitro*.

Apoptotic fragment number per crypt cell	Neutron dose(Dn) required(Gy)[a]	γ -ray dose(Dr) required(Gy)[a]	RBE(Dr/Dn)
0.5	0.033	0.064	1.939
1.0	0.088	0.176	2.000
1.5	0.146	0.306	2.096
2.0	0.208	0.469	2.255

The RBE values were estimated from the numerical values of the linear components of the dose-response curves presented in figure 3. The cells were incubated in RPMI with 20% FCS during the whole experiment.
[a]Calculated from fitting linear-quadratic model.

Discussion

In the present study, to develop an *in vivo* model system to estimate the predicted dose of target organs, we investigated the absorbed dose of radiosensitive organ, RBE and the comparative effects of ^{60}Co γ-rays and neutrons in crypt cells by measuring frequencies of apoptotic cells. Here, we used several apoptosis detection methods such as early alterations of plasma membrane, minor morphological alterations of nucleus, formation of apoptotic bodies and fragmentation of DNA[17].

The analysis of apoptosis in the present study indicated that the apoptotic cell formation after irradiation was dose-dependent and also manifested our earlier observed morphological characteristics of apoptosis such as cellular shrinkage, marked condensation and margination of chromatin, and nuclear and cellular fragmentation in mouse hair follicles. Mice crypt cells were found to have similar morphological characteristics of apoptosis in both high- and low-LET radiation, and even at low doses, apoptotic fragments were formed rapidly by these radiations. Since apoptosis is known to be time-dependent, several techniques that detect apoptosis at different times were used. There were significant peaks of apoptosis induction 12 h after irradiation in hair follicles, 4 and 6h in crypt cells after high- or low- LET radiation[8,9].

The results presented here also showed that the apoptotic fragment assay could be efficiently used in the study of DNA damage in several radiosensitive cells such as hair follicles and crypt cells. The apoptotic response to high-LET radiation was more rapid than low-LET radiation, as shown by the numerical change of remaining apoptotic cells at specific times and doses: At low dose range, the number of apoptotic cells in crypt cells markedly increased in radiation dose dependent manner, while a flattening toward higher doses was observed at higher doses. This initial steep increase in apoptosis demonstrates that mice crypt cells are very sensitive to immediate radiation damage, in agreement with other reports[4,13,18,19]. Especially, the decrease in the number of apoptotic fragment, at doses above 5 Gy might have resulted from either engulfed and digested apoptotic bodies by neighboring healthy phagocytes or necrosis. The above results indicate that the apoptotic fragment assay can estimate rapidly the predicted dose if samples are obtained quickly after irradiation. Consequently, the apoptotic fragment assay has an excellent potential to evaluate radiation-related biological effects as a radiation biodosimetric indicator of the most sensitive radiobiological endpoint. It is highly reproducible, dose-related, and quantified for any dose and quality, although the apoptotic fragments start to disappear, beginning on 4-12 h after irradiation, depending on cell types. The endpoint of investigation of apoptotic fragments is to detect subtle differences related to dose and radiation quality. With the presently decided novel apoptotic fragment assay, the tedious estimation of the predicted dose in the target organs of victims after accidental radiation exposure is no longer necessary, since the apoptotic fragment assay can predict much more rapidly with the radiosensitive cells like hair follicles and crypt cells. This assay can be used without dose estimation delay for screening radioprotectors, sensitivity of inter-cells, indicator of radiotherapeutic treatment of patients and co-parameter of biodosimetry, and also has the advantage to estimate the predicted dose early for initial medical treatment.

Quantification of apoptotic fragments is more sensitive, rapid and accurate than the other scoring systems which are based on visual observation[6,9,20,21]. In addition, this assay can also be used for the radiosensitivity, regardless of species, strains, organs and cells.

There are a few reports which compared radiation modalities of different LET for the induction of apoptosis. Damages

either to DNA or to cellular membranes after irradiation have been implicated, however, our own studies on neutron-induced crypt cells suggested that the site of principal damage was largely independent of LET: Regardless of radiation qualities, the quantitative change of the apoptotic fragment was produced by various doses. The time course for appearance and disappearance of apoptotic fragments were similar in both high-LET and low-LET radiation. However, significantly higher RBE for the induction of apoptosis at 6 h after irradiation was observed with high-LET radiation than with low-LET radiation. There was slight difference of RBE values between doses of both high-LET and low-LET radiation in crypt cells, but it was not significant. It should be mentioned here that previous work demonstrated conflicting results on RBE of apoptosis: The RBE of 1.0 for the induction of apoptosis in mouse thymocytes or for the induction of interphase death in lymphoid cells was reported using flow cytometric analysis, but our calculated RBE values for doses of 0.25 to 1 Gy were 2.072 in mice[4,22]. These values are very close to our previous data of micronucleus induction and chromosome aberration in our cyclotron fast neutrons[21,22]. Therefore, these conflicting results prompt us to study further in order to better understand the biological effects involved in apoptosis by differing LET radiation.

Finally, detailed information on humans would be required for radiation quality, dose rate, dose distribution, species, sex, and age although the apoptotic fragments assay should be used *in vivo* model system which show strong dose-dependence exhibiting easily quantifiable changes at low dose range.

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방사선에 전신 조사된 마우스 음와 세포의 아포토시스 유도를 이용한 생물학적 선량 측정 모델 개발 연구

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초 록 : 방사선 피폭선량의 예측을 위한 방사선 민감 지표 모델 개발의 일환으로 apoptotic fragment assay 법이 방사선에 피폭된 후 체내 피폭선량을 예측할 수 있는 지표로의 이용 가능성을 평가하기 위하여 코발트-60 감마선과 의료용 싸이크로트론 50MeV($p \rightarrow Be^{+}$) fast neutron 을 0.25Gy에서 1Gy의 선량을 마우스에 각각 전신 조사한 후 소장 음와세포내 apoptotic crypt cell의 수적 변화를 관찰하였다.

저선량 조사군에서 apoptotic crypt cell의 출현 빈도가 1Gy까지 급격하게 증가한 것으로 보아 방사선이 stem cell 지역에 있는 crypt cell의 형태학적 변화를 유발하는 것으로 나타났다. 이상의 결과는 아포토시스가 손상된 세포를 제거하므로 손상된 방사선 민감 표적 장기의 항성성 유지에 중요한 역할을 하는 것으로 판단되었다.

Apoptotic fragments의 발생빈도에 대한 선량-반응 곡선에 있어서 음와세포는 중성자조사군이 $y = 0.18 + (9.728 \pm 0.887)D + (-4.727 \pm 1.033)D^2$ (r^2=0.984) 으로, 반면에 감마선조사군은 $y = 0.18 + (5.125 \pm 0.601)D + (-2.652 \pm 0.7000)D^2$ (r^2=0.970)의 식을 얻었다. 이와 같이 중성자조사군과 감마선조사군은 공히 linear quadratic model 로 관찰되었다. apoptotic fragments 의 발생빈도와 조사 선량간에 유의한 효과가 있는 것으로 확인되었다.

이상의 결과에서 조사선량의 증가에 비례하여 방사선 민감 세포의apoptotic fragments 가 수적으로 증가하였으며, 고준위 방사선과 저준위 방사선은 선량 반응 관계식과 시간 경과에 따른 영향이 매우 유사하였으며, 마우스 음와세포의 apoptosis 유도에 대한 중성자선의 방사선 생물학적 효과비(RBE)는 2.072이였다. 그리고 모든 방사선조사군에서 방사선피폭 후 4시간과 6시간에 apoptosis 유도가 가장 많았으며, 음와세포의 형태학적 소견은 정상 대조군에서 관찰되지 않는 전형적인 apoptotic fragments 가 나타났다.

따라서 음와 세포에서의 아포토시스 유도는 방사선 피폭으로 발생된 세포 손상의 생물학적 영향 평가 검색, 방사선 방호제의 민감도 검사, 방사성 동위원소의 체내 오염에 대한 체내 피폭선량 예측의 지표 및 방사선 민감 표적장기의 손상정도 파악에 이용 가능할 것임. Apoptotic fragment assay 법은 0.25Gy에서 1Gy 까지의 선량에서 간편하고 빠르며 재현성이 있는 지표로서 방사선 민감 표적 장기의 선량 반응 평가와 방사선 피폭후 조기 피폭선량 예측을 위한 방사선 생물학적 선량측정법의 좋은 지표로 사용할 수 있을 것으로 사료됨.