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The apoptotic fragment assay in rat peripheral lymphocytes and crypt cells with whole body irradiation with ^{60}Co γ-rays and 50 MeV cyclotron fast neutrons

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Abstract : Here, we compared the effectiveness of 50 MeV(p→Be^{+}) cyclotron fast neutrons versus ^{60}Co γ-rays by the apoptotic fragment frequency in both rat peripheral lymphocytes and crypt cells to check a radiobiological endpoint. The incidence of apoptotic cell death was increased in all irradiated groups, and radiation at all doses trigger rapid changes in both crypt cells and peripheral lymphocytes. 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 these data of apoptotic fragments frequencies was $y = 0.3 + (6.512 \pm 0.279)D$ ($r^2 = 0.975$) after neutrons, while $y = 0.3 + (4.435 \pm 0.473)D + (-1.300 \pm 0.551)D^2$ ($r^2 = 0.988$) after γ-rays. In addition, $y = 3.5 + (118.410 \pm 10.325)D + (-33.548 \pm 12.023)D^2$ ($r^2 = 0.992$) after γ-rays in rat lymphocytes. 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 cells with increasing dose. Dose-response curves for high and low linear energy transfer (LET) radiation modalities in these studies were different. The relative biological effectiveness (RBE) value for crypt cells was 1.919. 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 induction in both crypt cells and peripheral lymphocytes could be a useful endpoint of rat model for studying screening test and microdosimetic indicator to evaluate the biological effects of radiation-induced cell damage.

Key words : Intestinal crypt cell, peripheral lymphocytes, apoptotic fragment assay, low dose radiation, biodosimetric animal model

Introduction

Numerous biological indicators have been proposed for dose estimation and prediction of late effects after radiation exposure. Biodosimetry of victims after acute radiation provides the best means of estimating radiation risk by extrapolating its biological effects to lower doses. Determination of the radiation exposure history of the general population for the prediction of health effects, risk assessment, and radiation protection has become increasingly important in the study of the biological effects at low dose range: a major obstacle to this goal of radiation dosimetry is the difficulty in establishing dose-response relationships *in vivo* for individuals because data of low levels are difficult to obtain. There are difficulties in reconstructing a valid biologically relevant dose and in assessing appropriate outcomes. Most biological indicators are not measures of accumulated doses but indications of biologically significant doses. Furthermore, biological dosimetry is not without its limitations; it is transient, technically difficult, and of limited sensitivity. Thus dose estimates and biomonitering are important components of risk assessment: it can provide means of triage for determining therapeutic strategies and prognoses. Biological dosimetric techniques provide a measure of the biologically relevant dose and involve the study of chromosome aberrations in blood lymphocytes after radiation exposure: biodosimetric model

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based on biological indicators to radiosensitive cells after accidental overexposure to radiation has been developed; in view of the growing importance of a reliable indicator to evaluate cell damages, information on the absorbed dose and its distribution is of great important for an early dose prediction of radiation consequences after accidental exposure. The best reliable indicator to evaluate the radiation biological effects required the development of rapid, simple and low cost techniques: 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: dose estimation by the calculated *in vivo* model system will help scientists to predict the absorbed dose of victims after radiation accident[1-7].

Many different cytogenetic and physical endpoints have been developed: physical dosimetry like electron spin resonance (ESR) and chromosome aberration has been known to be the promising endpoint for accidental exposure; chromosome aberrations, however, is time consuming and requires skilled technical support. ESR is used to detect free electrons produced by radiation in dental enamel and clothing of victims and is a good surrogate of absorbed dose, but provides no information about biological impact; it is not a direct measure of whole-body dose; it is subject to confounding factors such as the effect of ingested β-emitters. The problem of intersample variability in radiation sensitivity must also be addressed in ESR. ESR is limited in sensitivity and requires a large array of laboratory equipment and extracted teeth if dental enamel is to be used. Therefore, it is difficult for any indicator to measure the biological damages with confidence and subsequent risk associated with radiation exposure at low dose range[6-20].

Consequently, to develop animal model system of an alternative and simple biodosimetric technique that facilitates post-exposure calibration with greater flexibility, improved sensitivity and retrospective without the above-mentioned problems, we have 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. Apoptosis is an essential phenomenon in controlling cell and tissue homeostasis, and the relative degree of apoptosis has been shown to correlate with *in vivo* sensitivity to radiation. 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, 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. There is not any model system on the dose response relationship for crypt cell induced by low dose irradiation with ^{60}Co γ-rays and fast neutrons cyclotron because of variability and uncertainty along with other factors[21-24].

In the present study, the experiment was designed to obtain the effectiveness of fast neutrons versus γ-rays with respect to apoptosis induction in rat crypt cells and lymphocytes after whole body irradiation with ^{60}Co γ-rays and fast neutrons in order to examine whether the apoptotic fragment assay could be an indicator of biological dosimetry for the dose estimation of radiosensitive target organ damages.

Materials and Methods

Experimental animals

One hundred of Fisher 344 (Laboratory of Experimental Animal, Korea Cancer Center Hospital, KCCH) (7 to 8 weeks old, 300-320 g, respectively) 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.

Isolation of Lymphocytes and cell culture

Heparinized whole blood was obtained through rat abdominal vein, and lymphocytes were separated from freshly buffy coats after density gradient separation in Ficoll-paque(Pharmacia Fine Chemicals, Uppsala, Sweden) and washed twice in Hanks balanced salt solution. After washing, lymphocytes were fixed with Carnoy's solution, spreaded on cleaned slide and dried at 37°C in a humidified atmosphere.

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 rats 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 cut longitudinally and most of the lumen was selected for analysis. Employing distinctive morphological feature of apoptosis, as described by Kerr *et al*[25], 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 at various times 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 crypt cells after radiation exposure

To find the dose-response relationship after γ-rays and fast neutrons, the numbers of apoptotic fragments were counted in crypt cells and lymphocytes by light microscope (LM), respectively: the number of radiation induced apoptotic 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 and lymphocytes, as shown in Figs 1, 2.

The average numbers of apoptotic crypt cells and lymphocytes induced by γ-rays and fast neutrons, obtained by pooling the LM data of the 20 rats, are presented as a function of radiation dose and the error

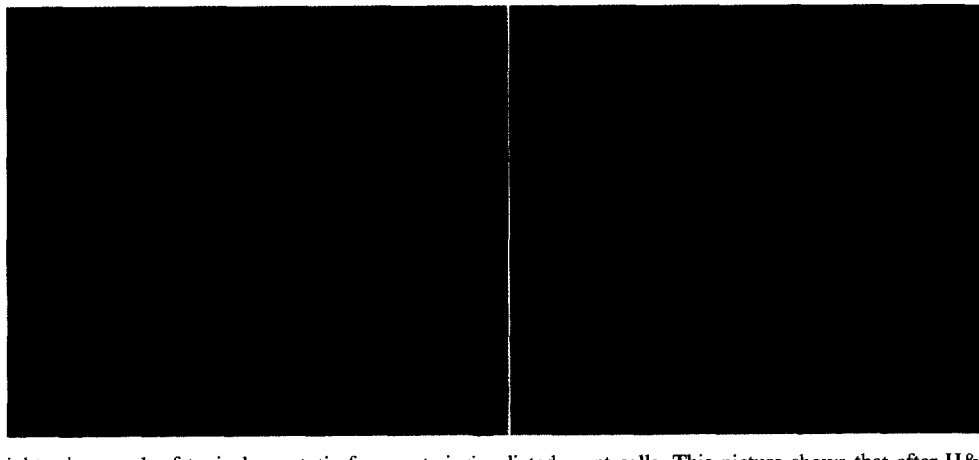

Fig 1. Light micrograph of typical apoptotic fragments in irradiated crypt cells. This picture shows that after H&E stain(A) and TUNEL assay(B) apoptotic fragments are distinguished. These cells are scored as 'apoptotic' (arrow heads).

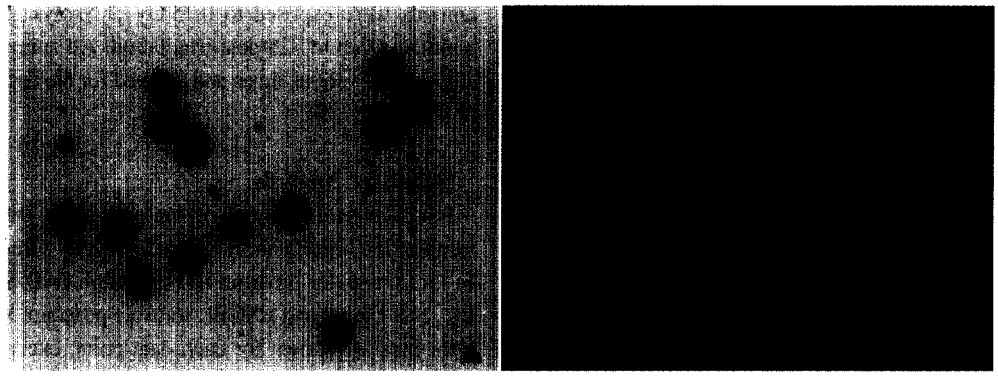

Fig 2. Micrographs of typical apoptotic cells in rat peripheral lymphocytes with H&E stain after irradiation with ^{60}Co γ-rays : A(x10), B(x40), apoptotic cells (arrow).

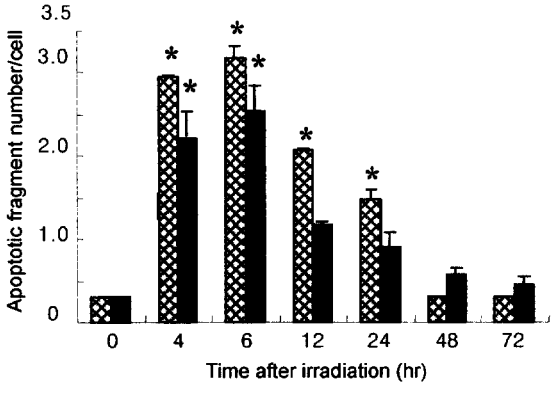

Fig. 3. Comparative biological effects of ^{60}Co γ-rays(■) and neutron (▩) for specific times as revealed from studies in rat crypt cells exposed *in vitro*.

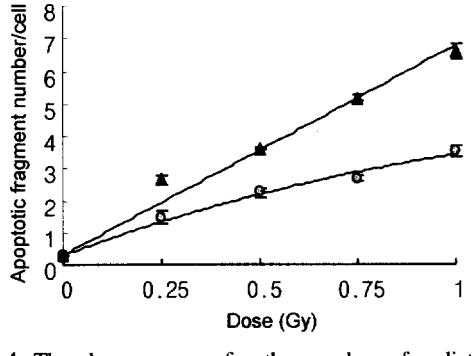

Fig 4. The dose-response for the number of radiation-induced apoptotic crypt cells stained in H&E, TUNEL assay, and DAPI 6h after irradiation with ^{60}Cr γ-rays (○) or fast neutron (▲). Compiled data from 20 rats where the mean ± SD for each dose are shown. A polynomial regression function was used to produce the linear quadratic curves from experimental data.

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 (Figs 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 4 indicate that the apoptotic process in high LET radiation was faster than that in low LET radiation. However, the spontaneous apoptotic frequency in the unirradiated groups showed no significant difference between individuals. The baseline number of apoptotic cells in unirradiated animal was low, being 0.3 in crypt cells and 3.5 in lymphocytes (Figs 2, 3).

Dose-response relationship

To evaluate the dose-response curves, the number of apoptotic cells was examined at the different doses, and the dose-response curve of apoptotic cells was obtained by fitting the linear-quadratic model $y = a+bD+cD^2$, where y is the yield of apoptotic 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 $y = 0.3+(6.512\pm0.279)D$ ($r^2 = 0.975$) after neutrons, while $y = 0.3+(4.435\pm0.473)D+(-1.300\pm0.551)D^2$ ($r^2 = 0.988$) after γ-rays (Fig 4). In addition, $y = 3.5 + (118.410\pm10.325)D+(-33.548\pm12.023)D^2$ ($r^2 = 0.992$) after γ-rays in lymphocytes (Fig 5).

There was a significant relationship between the frequency of apoptotic cell and dose. The dose-response curves were linear for neutrons and linear-quadratic for γ-rays. These data show trends towards increasing apoptotic cell number with increasing dose.

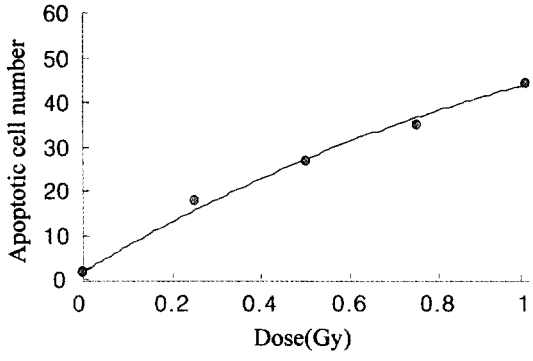

Fig 5. The dose-response for the cell number of radiation-induced apoptosis of rat peripheral lymphocytes 6 h after irradiation with ^{60}Co γ-rays. Analyses were performed after stained in H&E, TUNEL assay, and DAPI. Compiled data from 20 rats where the mean± SD for each dose are shown. A polynomial regression function was used to produce the linear quadratic curves from experimental data.

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 4 and Table 1, the mean RBE value for crypt cells was 1.919±0.085, 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.

Discussion

At present, there is no biodosimetric technique that can be used for monitering dose limits of low dose irradiation such as an occupational radiation exposure. This indicator or animal model system can definitely be found in the field of physical dosimetry. In case of radiation accidents involving high dose irradiation, several biological indicators such as conventional chromosome aberration and micronucleus in cytogenetic indicators are available, but there is no any indicator or animal model system to estimate the absorbed dose of internal target organs. One of the most prominent prerequisites of the biological model system used in dose reconstruction and prediction is its ability to estimate radiation dose for many samples within short time. Most such researches are based on the analysis of cytogenetic indicators in peripheral lymphocytes. There are abundant reports in the scientific literature that present reasonable results for the absorbed dose of victims associated with radiation accident[3,4,6,19,26,27]. At present, in each case, the strengths and weaknesses of the biodosimetric techniques used are exploited. Thus, it may be most reasonable to establish quantification of the biologically relevant dose in radiation dosimetry through the various techniques. Apoptosis induction in peripheral lymphocyte and crypt cells to establish a reliable biodosimetric technique is discussed as the ideal indicators to estimate cell damages of target organs, less expensive and less time and less difficult alternative to the traditional scoring of cytogenetic indicators. Difficulties exist for assessing the absorbed dose of target organs after internal contamination and accidental exposure because of interinvidual variability and uncertainty along with other factors[5,6].

Means of predicting the risk to humans after radiation exposure is to develop 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 dose estimation. In the present study, to develop quantitative model system to assess the absorbed dose of target organs to aid the understanding of the accident

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

Apoptotic fragment number per crypt	Neutron dose(Dn) required(Gy)[a]	γ-ray dose(Dr) required(Gy)[b]	RBE(Dr/Dn)
0.5	0.030	0.056	1.867
1.0	0.107	0.202	1.888
1.5	0.184	0.356	1.935
2.0	0.261	0.519	1.989

The RBE values were estimated from the numerical values of the linear components of the dose-response curves presented in figure 6. The cells were incubated in RPMI with 20% FCS during the whole experiment.

[a]Calculated from fitting linear model

[b]Calculated from fitting linear-quadratic model

process and radiation doses and distribution after accidental exposure, we studied RBE for the absorbed dose of radiosensitive cells and the comparative effects of γ-rays and neutrons in both crypt cells and lymphocytes 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[23].

The analysis of apoptosis in our results 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 with our earlier data of mouse hair follicles. Rat crypt cells and lymphocytes were found to have similar morphological characteristics of apoptosis in both high- and low-LET radiation, and even at low doses, apoptotic fragments were observed easily 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 12h after irradiation in hair follicles, 4 and 6h in crypt cells and lymphocytes after high- or low- LET radiation[24,25]. After exposure to low dose irradiation, rat model system is most frequently to describe the dose-response relationship for apoptosis induction in lymphocytes and crypt cells.

The results presented here also showed that rat model system as a quantitative technique could be efficiently used in dose prediction of target organ through the study of DNA damage of radiosensitive cells such as lymphocytes and crypt cells. The apoptotic response of rat radiosensitive cells 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 both crypt cells and lymphocytes markedly increased in radiation dose dependent manner, while a flattening toward higher doses was observed at higher doses. These increases of apoptosis induction in crypt cells and lymphocytes demonstrate that rat model system is very sensitive to immediate radiation damage with our earlier data of hair follicles.

Accordingly, the above results indicate that in cases when an acute whole-body exposure has occurred and the screening of many victims is necessary, rat model system with the apoptotic fragment assay should be useful because the assay is very rapid and easy to estimate the predicted dose if samples are obtained quickly after irradiation. Consequently, the rat model system has an excellent potential to evaluate radiation-related human biological effects as a biodosimetric model system with the most sensitive radiobiological endpoint. Apoptosis induction in rat model system 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. With the presently decided novel biodosimetric assay system, the tedious estimation of the predicted dose in the target organs of victims after accidental radiation exposure is no longer necessary, since apoptosis induction in rat model system can be predicted much more rapidly with the radiosensitive cells like lymphocytes, hair follicles and crypt cells. This model system 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 absorbed dose for initial medical treatment by the dose-response relationship. Therefore, this model system can also be used for the radio-sensitivity and biodosimetric assay regardless of species, strains, organs and cells.

Finally, detailed information on humans by comparing effects of rat model system would be required for radiation quality, dose rate, dose distribution, species, sex, and age although this model system should be used another alternative endpoint for dose prediction of radiosensitive target organs and the *in vivo* model system which show strong dose-dependence exhibiting easily quantifiable changes at low dose range.

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코발트-60 감마선과 50 MeV 싸이크로트론 고속 중성자선에 전신조사된 랫드의 말초 임파구와 음와 세포의 아포토시스 유도를 이용한 생물학적 선량 측정 모델 개발 연구

김 태 환

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국문초록 : 방사선 피폭선량의 예측을 위한 방사선 민감 지표 모델 개발을 위하여 코발트-60 감마선과 의료용 싸이크로트론 50 MeV($p \rightarrow RBe^+$) fast neutron을 0.25 Gy에서 1 Gy의 선량을 랫드에 각각 전신 조사한 후 말초혈액내 임파구와 소장내 음와세포의 형태학적 변화를 apoptotic fragment assay 법을 이용하여 관찰하였다. 모든 방사선조사군에서 음와세포와 말초 임파구에 아포토시스의 유도가 증가된 것이 관찰되었으며, 이것은 방사선이 방사선 민감세포의 아포토시스 유도를 자극한 것으로 보인다. 상기의 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 중성자 조사군이 $y = 0.3 + (6.512 \pm 0.279)D (r^2 = 0.975)$으로, 반면에 감마선 조사군은 $y = 0.3 + (4.435 \pm 0.473)D + (-1.300 \pm 0.551)D^2 (r^2 = 0.988)$의 식을 얻었다. 그리고 말초 임파구에서는 감마조사군이 $y = 3.5 + (118.410 \pm 10.325)D + (-33.548 \pm 12.023)D^2 (r^2 = 0.992)$의 식으로 나타났다. 이와 같이 감마선조사군은 공히 linear quadratic model 이였으나 중성자조사군은 linear model로 관찰되었다. 조사된 세포의 종류와 상관없이 apoptotic fragments의 발생빈도와 조사 선량간에 유의한 효과가 있는 것으로 확인되었다. 이상의 결과에서 조사선량의 증가에 비례하여 방사선 민감 세포의 apoptotic fragments가 수적으로 증가하였으며, 고준위 방사선이 저준위 방사선보다 선량 반응 곡선과 시간 경과에 따른 영향이 보다 강한 것으로 인지되었으며, 음와세포의 apoptosis 유도에 대한 중성자선의 방사선 생물학적 효과비(RBE)는 1.919 였다. 그리고 모든 방사선조사군에서 방사선피폭 후 4시간과 6시간에 apoptosis 유도가 가장 많았으며, 음와세포의 형태학적 소견은 정상 대조군에서 관찰되지 않는 전형적인 apoptotic fragments가 나타났다. 따라서 음와 세포와 말초 임파구에서의 아포토시스 유도는 방사선 조사에 의한 세포 손상의 생물학적 영향 평가를 위한 검색 및 방사선 피폭선량 예측의 지표로 이용 가능할 것으로 사료됨.

Key words : Intestinal crypt cell, peripheral lymphocytes, apoptotic fragment assay, low dose radiation, biodosimetric animal model