Quantitative Analysis of DNA Single-strand Breaks in EL 4 cells and Mouse Spleen Lymphocytes after Irradiation

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The filter elution technique was used to assay Co-60 γ ray-induced DNA single-strand breaks (SSB) in EL 4 mouse leukemia cell and mouse spleen lymphocyte.

The lymphocytes were stimulated with lipopolysaccharide (LPS, 20 μg/ml) to label [^{3}H] thymidine. EL 4 cells and lymphocytes in suspension were exposed at 0°C to 0 Gy, 1 Gy, 5 Gy, 10 Gy of Co-60 radiation and elution procedure was performed at PH 12.1.

The number of DNA single-strand breaks increased with increasing doses of γ rays. The strand scission factor (SSF) was estimated in each experiment (eluted volume 21 ml). The slope for EL 4 cells was 0.01301 ± 0.00096 Gy^{-1}($n=5$) and the slope for lymphocytes was 0.01097 ± 0.00091 Gy^{-1} ($n=5$). The slopes were significantly different ($P<0.005$). Thus EL 4 cells were more sensitive to induction of DNA SSB by ionizing radiation than lymphocytes.

Key Words: Alkaline elution, EL 4, Lymphocytes, Single strand breaks, Filter

INTRODUCTION

As radiation biology has developed, many experimental methods which explained the mechanisms of specific radiobiological characteristics induced by irradiation have been innovated. Up to date many researches for elucidating diverse radiobiological effects such as cell death and chromosomal aberration have been performed, but explanation of direct interrelationship between these phenomena was very difficult. Furthermore, irradiation of beam of different quality, change in dose rate, change in oxygen content, and administration of radioprotector or radiosensitzer can induce variable reactions even in the same cells[1~6].

DNA is considered as a major target of radiation in the living cells and DNA damage results in inactivation of ability of cell proliferation, mutation, and chromosomal aberration[2,7~9]. Radiation effects on soluble DNA, dry DNA, viral DNA and cellular DNA have been reported by many persons[7].

Discontinuity in the nucleotide strand is one of the most severe damage by radiation in the ultrastructure of DNA and these strand breaks occur in the one of double helix (single strand breaks: SSB) or both (double strand breaks: DSB). Strand breaks induced by ionizing radiation result in functional damage of DNA, that is, diminished activation of DNA for RNA synthesis or loss of transcription ability of DNA[7]. Some scientists reported that strand breaks by irradition could be repaired rapidly in the living cells[10,11].

Therefore, to grasp the essential features of DNA strand breaks induced by radiation is a prerequisite for understanding the radiation effects on cells as well as responses of enzymes participating in repair process.

The objectives of the present experiment are as follows.

1. To investigate the radiation effects of Co-60 γ-ray on EL 4 leukemia cells originated from C57BL/6 mouse and spleen lymphocytes from syngeneic mouse, especially single strand breaks (SSB).
2. To improve the methods carried out up to date and make them simpler and more practical.
3. To obtain the data for more diverse application of our experimental design.

MATERIALS AND METHODS

1. Separation of Normal Lympocytes and Radioisotope labelling (Fig. 1)

Spleens were extracted from 6 weeks-aged Sprague-Dawley rats and 6 weeks-aged C57BL/6 mice. After washing with Hank's balanced salt solution (HBSS), they were minced in the petri-dish containing 10 ml of HBSS with resultant cells

suspension. Suspended cells were added on the Ficoll-hypaque solution and centrifuged at 400 gravity for 30 minutes. Lipopolysaccharide (LPS) of 20 μg/ml was added to 5×10^5 cells/ml of separated cells and we measured the cellular proliferation by micro-culture assay for 24, 48, and 72 hours respectively. [^{3}H] thymidine (3.0 TBq/mmol, 81.9 Ci/mmol) of 2 μCi/ml was added 4 hours prior to termination of culture.

2. Cells Used in Experiment

EL 4 mouse leukemia cells and spleen lymphocytes of C57BL/6 mice, of which cellular proliferation was stimulated easily in the presence of LPS, were used. Each cell was cultured on RPMI 1640 media supplemented with 10% fetal bovine serum (FBS), 100 unit penicillin, 10 μg streptomycin, and 2 mM L-glutamine. Uniform labelling of DNA was obtained in EK 4 leukemia cells with [^{3}H] thymidine of 0.2 μCi to 1×10^6 cells/ml after 16 hours' culture and mouse spleen lymphocytes were cultured for 4 hours, 44 hours after adding LPS to 5×10^5 cells, with 2 μCi. Each labelled cell was used in the experiment after having cultured in the label-free medium.

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Spleen
↓
Mincing
↓
Single cell suspension
↓
Layering ..................... Ficoll-paque sol.
↓
Isolation of lymphocyte ... centrifuge 400 g, 30-40 min.
↓
Washing ...................... HBSS x 3
↓
Cell counting
↓
Culture ...................... 1 x 10⁶ cell/ml
                               RPMI 1640
↓                              mitogen (LPS)
                               37 °C, 5 % CO₂

Labelling .................... [³H]thymidine, 2 μCi/ml
                               4 hrs. before cell harvest
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Fig. 1. Isolation of normal lymphocyte and labelling of [^{3}H]TdR.

3. Irradiation

Radio-labelled EL 4 cells and lymphocytes with [^{3}H] thymidine were stained with trypan blue and their survival rates were measured with hemocytometer. Afterthen, Each 1×10^6 cells was put into 4 well of 24 well bottom culture plate (Flow lab. Inc.), followed by irradiation on the ice. Co-60 γ-ray of 1, 5, 10 and 15 Gy were delivered by Theratron-780 teletherapy unit with 95.0 rad/min, immediately followed by 10-fold dilution of cell suspension by frozen phosphate buffered saline (PBS).

4. Alkaline Elution (Fig. 2)

The standard procedure used was as follows (modified from Kohn's method[12].) Cell suspen-

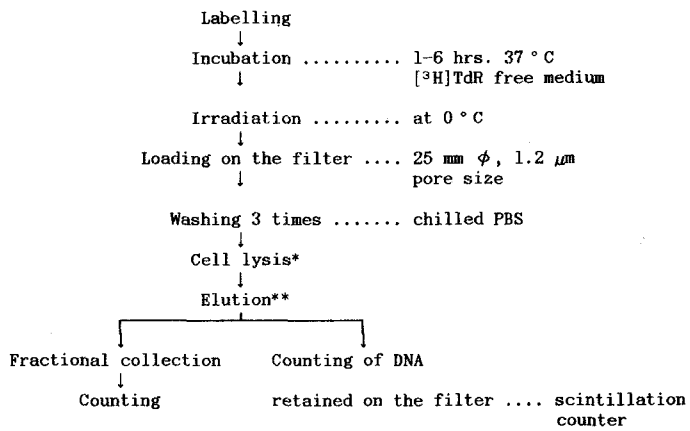

Fig. 2. DNA strand breaks assay procedure.

sions were diluted 10-fold with cold PBS, filtered onto a 25 mm diameter, 1.2 μm pore size polyvinyl chloride filter (Gelman Sciences Inc.), and washed 3 times with cold PBS. The cells were lysed on the filter at room temperature with 5 ml of lysing solution [0.05 M Tris (base), 0.05 M glycine, 0.025 M EDTA (disodium salt), 2% w/v SDS, 0.5 mg/ml proteinase K], pH 9.6 (By method of Koval and Kazmar [13]). The flow of lysing solution was stopped when reached 1 ml, incubated for 30 minutes at room temperature, and washed twice with 0.02 M EDTA solution, pH 10.3. The DNA was subsequently eluted in the dark with addition of 25 ml of the alkali elution solution, pH 12.1 (lysis solution minus proteinase K) at a constant flow rate of 0.01 ml/minutes by using multi-channel pump (Manostat cassette pump). Fractions were collected every 30 minutes for 300 minutes (10 times), resulting in a fraction volume of approximately 3.0 ml. The filter was removed from filter holder and DNA remaining in the filter holder apparatus was recovered by flushing vigorously with 3 ml of 0.4 M NaOH solution. Any DNA retained on the filter at the end of the elution time was recovered by hydrolytic depurination for 1 hour at 60°C in 1 M HCl (0.4 ml) followed by the addition of 0.4 M NaOH (2.5 ml) for 30 minutes at room temperature to convert the apurinic sites to strand breaks. The amount of DNA in each fraction, as well as that remaining on the filter and that recovered from the interior of the filter holder, was assayed by liquid scintillation counting.

5. Liquid Scintillation Counting

Each 1 ml of lysing solution, elution solution and washing solution of filter holder was collected. Ten milli-liter of scintillation cocktail (lumagel, lumac, Netherlands) was added on the filter and radioactivity, then, was measured with scintillation counter (Packard Co.)

6. Calculation of Strand Scission Factor (SSF)

The relative number of strand breaks was determined by equation, $SSF = -\log (fx/fo)$, where fo and fx are, respectively the proportion of DNA retained on the filter for the unirradiated control and for the irradiated sample. For SSBs, the values for fraction of DNA retained at an eluted volume of 21 ml were used for the calculation. Analysis of SSF versus dose yielded linear response curves for SSF at 20 ml and gave better reproducibility than at lesser volumes. The data are the average of at least six separate experiments, and the standard error is displayed with the mean.

RESULT

1. Proliferation of Normal Lymphocytes (Table 1)

Effect on cellular proliferation by LPS was minimal in the spleen lymphocyte of rat and maximum 48 hours before culture in the lymphocyte of C57BL/6 mice. In our experiment were used spleen lymphocytes derived from C57BL/6.

2. Single-Strand Breaks (Table 2 and 3)

Typical alkaline elution profiles for DNA from mouse cells after whole-body exposure to various doses of ionizing radiation are shown in Fig. 3 and4. The profiles shown are for EL 4 leukemia cells and normal spleen lymphocytes, the 2 cell lines demonstrating moderate differences in sensitivity to radiation in terms of the formation of SSB in vitro. Dose-response curve indicating the degree of DNA strand scission in relation to the dose of radiation was presented in Figure 6 and fitted with straight lines by linear regression analysis and the slopes were calculated. Their values were 0.01301 ± 0.00096 Gy^{-1} ($n=5$) in EL 4 leukemia cells and 0.01097 ± 0.00091 Gy^{-1} ($n=5$) in the normal spleen lymphocytes. These facts showed that the efficiency of SSB formation was greater in EL 4 leukemia cells than in normal spleen lymphocytes when irradiation was performed in vitro ($P<0.005$). Figure 5 showed that percent DNA damage was

Table 1. Lymphoproliferative Responses by Lipopolysaccharide

Incubation time (hours)	C57BL/6 mouse		Sprague–Dawley rat	
	None	20 μg/ml	None	20 μg/ml
24	2218.6 ± 159.5*	82301.2 ± 2531.8	763.1 ± 41.7	11511.5 ± 100.9
48	4414.1 ± 962.0	340087.3 ± 28776.1	1817.0 ± 128.3	32667.1 ± 744.8
72	4190.2 ± 657.7	135918.8 ± 10813.4	486.8 ± 78.0	4600.2 ± 536.5

* Mean ± S.D. of the counts per minute of tritiated thymidine incorporation by triplicate microcultures of 1×10^5 normal lymphocytes.

Table 2. Percentage of DNA in Each Fraction to Total Amount of DNA from γ Ray Irradiated EL 4 Cell

Groups	Number of fraction*									
	1	2	3	4	5	6	7	8	9	10
Control	7.75 ±1.06**	4.64 ±1.1	4.82 ±1.58	2.89 ±0.52	1.94 ±0.92	2.44 ±1.39	1.5 ±0.74	2.52 ±2.29	1.62 ±1.08	1.16 ±0.63
1 Gy	7.78 ±5.07	6.72 ±1.58	6.42 ±2.36	3.39 ±1.79	2.61 ±1.47	1.95 ±0.86	1.24 ±0.89	2.14 ±0.61	1.57 ±0.55	2.55 ±2.02
5 Gy	10.38 ±4.8	8.03 ±0.68	5.53 ±4.32	3.29 ±1.98	2.22 ±1.35	3.18 ±1.59	2.25 ±1.1	2.69 ±2.08	1.48 ±0.78	1.21 ±0.5
10 Gy	10.05 ±3.75	8.81 ±3.45	6.56 ±2.89	5.78 ±1.69	3.44 ±0.29	3.33 ±0.65	3.8 ±2.62	3.53 ±1.07	2.96 ±2.22	2.58 ±1.57
15 Gy	26.99 ±2.43	8.1 ±2.03	5.91 ±1.19	3.29 ±1.07	3.37 ±0.83	3.68 ±1.18	3.56 ±0.45	3.03 ±0.45	4.58 ±0.72	3.23 ±0.32

* Fractions were collected every 30 minutes at a flow rate of 0.1 ml/minute. ** Mean ± S.D.

Table 3. Percentage of DNA in Each Fraction to Total Amount of DNA from γ Ray Irradiated Mouse Lymphocyte

Groups	Number of fraction*									
	1	2	3	4	5	6	7	8	9	10
Control	5.04 ±0.29**	2.95 ±1.0	1.79 ±0.3	1.34 ±0.37	2.03 ±1.24	1.14 ±0.21	0.77 ±0.26	0.75 ±0.12	0.99 ±0.63	0.8 ±0.5
1 Gy	5.68 ±0.42	4.98 ±1.05	2.42 ±0.21	1.76 ±0.06	1.47 ±0.14	1.5 ±0.57	1.23 ±0.51	0.85 ±0.1	1.09 ±0.44	0.87 ±0.29
5 Gy	8.35 ±0.52	6.01 ±0.69	3.61 ±0.47	2.46 ±0.43	1.8 ±0.17	1.29 ±0.08	0.96 ±0.1	0.74 ±0.09	0.8 ±0.24	0.76 ±0.29
10 Gy	9.47 ±0.93	7.49 ±0.4	5.13 ±0.49	3.08 ±0.1	2.12 ±0.23	1.51 ±0.17	1.01 ±0.12	1.09 ±0.2	0.63 ±0.06	0.63 ±0.06
15 Gy	12.62 ±1.55	9.12 ±1.63	8.78 ±2.4	6.03 ±1.75	4.0 ±1.5	2.26 ±0.52	1.42 ±0.31	1.62 ±1.03	1.82 ±1.59	0.88 ±0.13

* Fractions were collected every 30 minutes at a flow rate of 0.1 ml/minute. ** Mean ± S.D.

higher in EL 4 leukmia cells, although unirradiated, than in the normal lymphocytes, suggesting that there may be enzyme deficiency taking part in repair of single-strand breaks or the presence of certain factors inhibiting activity of repair enzyme in the malignant cells.

DISCUSSION

Cell death induced by exposure to ionizing radiation is considered as a consequence of DNA damage. To determine the degree of single-strand breaks of DNA with alkaline elution technique provides us a piece of information about radiation damage to cells. Therefore, measurement of DNA strand breaks is a valuable experimental method from the standpoint of understanding the DNA damage and repair mechanism from it[15]. The methods measuring the single-strand breaks of DNA have been developed diversely--alkaline unwinding[16], alkaline filter elution[17], nucleotide sedimentation[15], viscoelastrometry [18], microelectrophoresis of single cell[19], DNA precipitation[15], pulse gel electrophoresis[20], fluctuation spectroscopy[21] and nick translation[22].

Advantages of the alkaline elution technique[12] include: (a) its sensitiveity (the DNA strand breaks can reproducibly be detected after radiation doses as low as 1.00 gray) and (b) that many samples, up to 16 on one pump and fraction collector, can be run at the same time (4). Although its inability to generate accurate estimations of DNA molecular weight, alkaline elution has proven to be an extremely effective method for measuring the

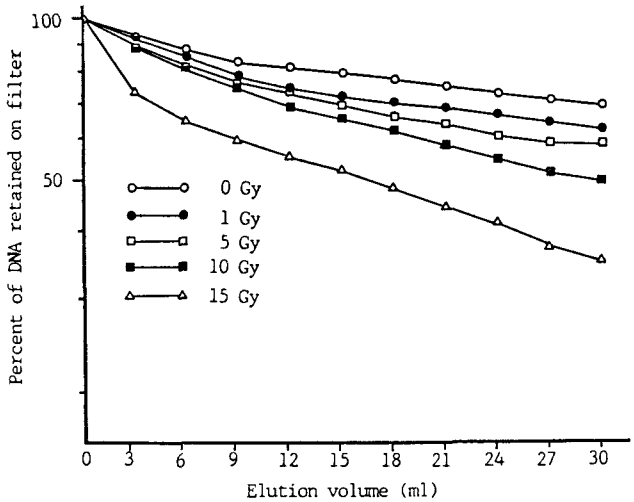

Fig. 3. Percent of EL 4 leukemia cell DNA retained on the filter versus elution volume. The cells in suspension were exposed at 0°C to each dose of Co-60 radiation

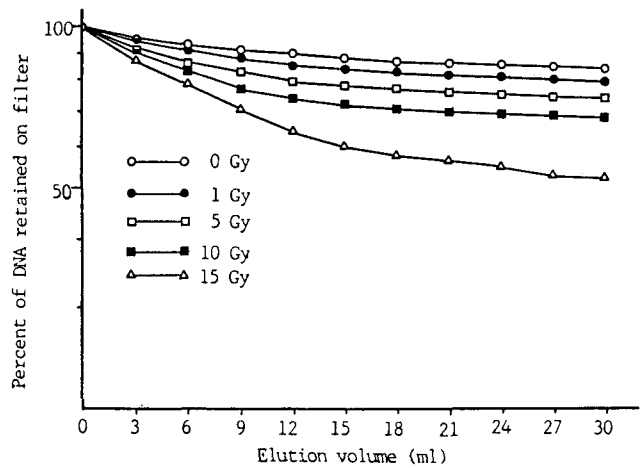

Fig. 4. Percent of mouse spleen lymphocyte DNA retained on the filter versus elution volume. The cells in suspenison were exposed at 0°C to each dose of Co-60 radiation

relative amount of several different types of DNA damage in mammalian cells exposed to a variety of agent.

The elution techniques utilize filters to discriminate DNA sizes in mammalian cells. The filters do not absorb DNA under conditions employed, but act mechanically to impede the passage of DNA. These techniques can be used to measure single-strand breaks, double-strand breaks, alkali-labile sites, DNA-protein crosslinks, and DNA interstrand crosslinks[15].

Meyn and Jenkins[4] measured the efficiency of DNA strand break formation in normal and tumor tissues of mice by using the technique of alkaline elution coupled with a microfluorometric determination of DNA. According to their results, substantial differences existed among various tissues in terms of the amount of DNA strand break damage produced for a given dose of radiation. Of normal tissues, the most breaks were produced in bone marrow and the least were produced in gut. Strand break production was relatively inefficient

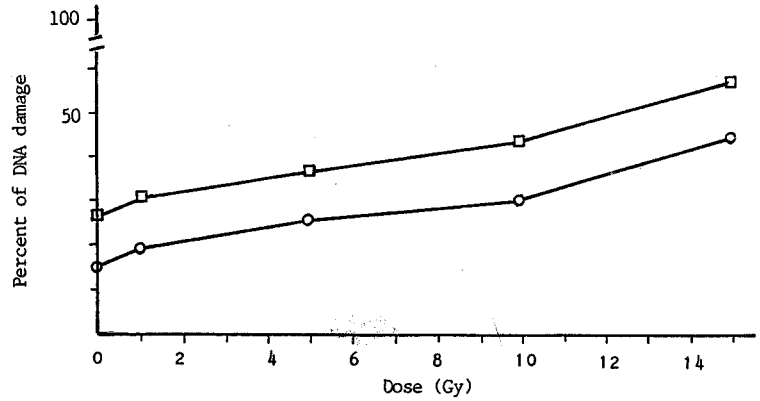

Fig. 5. Percent of DNA damage for EL 4 (□) and mouse lymphocyte (○) exposed at 0°C to each dose of Co-60 radiation.

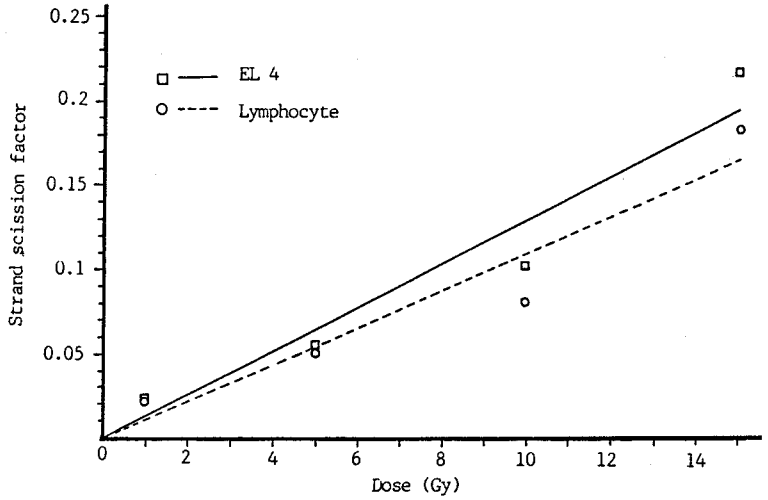

Fig. 6. Strand scission factor versus radiation dose for EL 4 leukemia cell and mouse lymphocyte.

in the tumor (Fibrosarcoma induced in C3H mice by methylcholanthrene) compared to normal tissues. These results are in a sharp contrast to our results. In our experiment, single-strand breaks were more efficient in tumor cells (EL 4 leukemia cells) than in normal lymphocytes. Perhaps this difference may be due to different cell lines used in experiments—that is, fibrosarcoma cell lines are inherently radioresistant and in contrary, EL 4 leukemia cell lines are more susceptible or sensitive to irradiation. Especially as showed in Figure 3, percent DNA damage was higher in EL 4 leukemia cell lines than in normal lymphocytes even in the unirradiated state.

The factors influencing the efficiency of DNA strand breaks formation are variable. By Meyn and Jenkins[4], the efficiency of DNA strand break formation measured in the cells from the tissues irradiated in vitro was much more uniform and considerably greater than that measured in vivo, suggesting that the normal tissues in the animal may be radiobiologically hypoxic.

Elution solution composition and pH of lysing and eluting solution also affect DNA strand breaks, reported by Koval and Kazmar[13].

We used perfusion solution made by Koval and Kazmar[13]. The composition of lysing solution is the same as that of elution solution, except addition of 0.5 mg/ml protein kinase K to the lysing solution. The advantages of this lysing solution include: (a)

simple preparation for experiments, due to similar component of these two solutions, (b) using of Tris-buffered lysing and eluting solution in which difference of elution rate between them is negligible according to pH change. We also shortened experimental period with perfusion rate of 0.01 ml/minutes.

The alkaline elution phenomenon provides an sensitive measure of a physical effect of ionizing radiation on DNA in mammalian cells and thus estimation of relative radiation sensitivity by analysis of tumor cells by DNA elution might be useful as a predictive assay of tumor response to therapy. It would be of interest to compare the sensitivities of tumor and normal cells, determined on the basis of the efficiency of SSB production, with their radiosensitivitites as measured by conventional methods, such as in vivo colony assays. Before using DNA elution as a predictive assay of tumor radiosensitivity, however, a number of factors need to be considered: (a) many tumor samples are limited in size and therefore the number of time points one can examine by elution is small, (b) DNA elution is a bulk assay; one cannot distinguish subpopulations of cells with this assay. A tumor biopsy obviously contains clonogenic as well as nonclonogenic cells and normal infiltrating cells as well as tumor cells. (c) the absolute yield of DNA strand breaks is not the sole determinant of radiosensitivity.

Our experiment in which the normal lymphocytes were separated from mice spleen and then irradiated in vitro was conducted to serve as controls for oncoming in vivo experiments. The EL 4 leukemia cells were more sensitive to radiation-induced strand break damage than the normal lymphocytes. While we can only speculate as to the meaning of this result, it seems possible that these tumor cells might be deficient in naturally occurring radioprotective mechanism in vivo or in vitro, such as nonprotein sulfhydryls or superoxide dismutase, compared to normal cells which might render them more sensitive when irradiated in air. In any event, the explanation for this suggestion will depend on further investigations.

We have a plan to investigate the characteristics of radiation and radiobiological effects on normal tissues and tumor cells in vivo by using DNA strand breaks elution technique. Furthermore, we will discriminate the effects of the existing and new radioprotectors on repair of radiation-induced DNA damage or radioprotection.

CONCLUSION

We measured the efficiency of DNA single-strand breaks formation of EL 4 leukemia cells and spleen lymphocytes derived from C57BL/6 mice, induced by γ-ray irradiation, with alkaline filter elution technique.

1. The amounts of single-strand breaks increased as the radiation doses increased in both EL 4 leukemia cells and normal lymphocytes: Linear dose-response curve

2. For single-strand breaks, the values for fraction of DNA retained at an eluted volume of 21 ml were used for calculation. Strand Scission Factor (SSF) in EL 4 leukemia cells was 0.01301 ± 0.00096 Gy^{-1} and 0.01097 ± 0.00091 Gy^{-1} in spleen lymphocytes, respectively.

3. EL 4 leukemia cells were more sensitive to radiation than normal lymphocyte in terms of DNA single strand breaks.

4. We can use alkaline filter elution technique as a predictive assay for radiosensitivity of normal and tumor cells in lieu of conventional clonogenic assays alternatively.

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= 국문초록 =

방사선에 의한 EL 4 백서 백혈병 세포 및 정상 백서 비장 임파구 DNA Single-Strand Breaks의 정량적 분석과 측정

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Filter elution 방법으로 EL 4 백서 백혈병 세포 및 C57BL/6 백서 유래의 비장 임파구에 대한 Co-60 γ 선의 DNA single-strand breaks (SSB) 효과를 정량적으로 측정하였다.

임파구는 [^{3}H]thymidine을 표지하기 위하여 lipopolysaccharide (LPS, 20 μg/ml)를 첨가하여 자극하고 부유 상태의 EL 4 세포 및 임파구를 0°C에서 0 Gy, 1 Gy, 5 Gy, 10 Gy 또는 15 Gy조사하였으며, elution 용액의 pH는 12.1로 하였다.

γ 선 조사에 따른 single-strand breaks의 수는 방사선 조사량에 따라 증가 하였으며 21 ml의 elution 양을 기준으로 한 strand scission factor (SSF)는 EL 4 세포에서 $0.01301 \pm 0.00096\ Gy^{-1}$ (n=5)이었고, 임파구는 $0.01097 \pm 0.00091\ Gy^{-1}$ (n=5)를 나타내므로 본 실험에서는 EL 4 세포가 정상 임파구에 비하여 방사선에 의한 DNA SSB가 민감함을 알 수 있었다($p<0.005$).

본 연구 결과 DNA strand breaks의 측정법을 이용하여 방사선의 특성 및 생물학적 효과의 파악은 물론 나아가 기존의 방호제 및 새로운 약제의 DNA에 대한 효과를 판별할 수 있을 것이다.