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Cytokinesis-blocked micronuclei in the human peripheral lymphocytes following low dose γ- rays irradiation

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Abstract : To determine if micronucleus (MN) assay could be used to predict the absorbed dose of victims after accidental radiation exposure, we carried out to assess the absorbed dose depending on the numerical changes of MN in human peripheral blood lymphocytes after ^{60}Co γ- rays exposure in the range of 0.25 to 1 Gy, respectively. The MNs were observed at very low doses, and the numerical changes according to doses. Satisfactory dose-effect calibration curve is observed after low dose irradiation of human lymphocytes *in vitro*. When plotting on a linear scale against radiation dose, the line of best fit was $Y = (0.02\pm 0.0009) + (0.033\pm 0.010)\,D + (0.012\pm 0.012)\,D^2$. The dose-response curve for MN induction immediately after irradiation was linear-quadratic and has a significant relationship between the frequencies of MN and dose. These data show a trend towards increase of the numbers of MN with increasing dose. The number of MN in lymphocytes that were observed in the control group is 0.1610± 0.0093/cell. Accordingly, MN assay in human peripheral lymphocytes could be a useful *in vivo* model for studying radio-protective drug sensitivity or screening test, microdosimertic indicator and radiation-induced target organ injury. Since MN assay is simple, rapid and reproducible, it will also be a biodosimetric indicator for individual dose assessment after accidental exposure.

Key words : micronuclei, lymphocyte, radiation biological dosimetry, radiation accident

Introduction

Many studies have been performed to assess the development and application of potentially useful biological dosimetry for the quantitative index of radiation exposure. Measurement of cytogenetic damage by simple and informative techniques would be of great value in studying genetic risk following occupational, therapeutic and accidental exposure to radiation. In circumstances if physical dosimetry is unavailable or unreliable, the extent of exposure can be estimated by the level of radiation-induced genetic damage. At present, this is usually performed by determining the frequencies of dicentric chromosomes in peripheral blood lymphocytes. Although this is a sensitive method for dose estimation, it is laborious and needed enough experience to estimate, and without automation its scope for population screening is limited. Especially, chromosome aberration has the limitation in assessing the irradiated dose in case of low dose exposure. Therefore, we need an alternative cytogenetic dosimetry to estimate the absorbed dose of victims after low dose exposure such as radiation accidents in hospital workers and workers of radiation related facilities[1-7].

An alternative and simple cytogenetic technique is the measurement of micronucleus frequency in cultured human lymphocytes. As micronuclei originate from both acentric fragments and whole chromosomes that have lagged behind at anaphase, or have been displaced from the metaphase, they provide a measure of both chromosome breakage and whole chromosome loss. The reliability of conventional micronucleus assays is diminished owing to the inclusion of non-dividing cells in the estimate, but this problem has been overcome by the development of cytokinesis-blocked(CB) micronucleus assay which enables micronucleus to be scored specifically in those cells that have completed nuclear division. The CB technique improves the reliability and sensitivity of the micronucleus

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assay because of being recognized by their binucleate appearance following inhibition of cytokinesis by cytochalasin-B(Cyt-B). The reliable and ease assays of the cytokinesis blocked-approach are obvious advantages in biological monitoring. Therefore, the lymphocytes would appear to offer the only system where dose distribution over the body surface might be assessed by an estimate of received dose in a suitable time-scale for clinical intervention. Although the peripheral lymphocytes are in Go phase, they are radiosensitive. The characteristics of the dose-response relationships at high doses obtained by the CB method after exposure *in vitro* to ionizing radiation have been evaluated by many different researchers, but few studies have been performed to measure chromosome damage in humans following low dose exposure to ionizing radiation and not developed recognizable and reliable techniques for biological dosimetry of low dose exposure until recently[8-11].

Accordingly, to determine the usefulness of CB micronucleus assay for investigating dose-response relationship of peripheral lymphocytes following low dose irradiation, the present study was performed to evaluate a dose-effect calibration curve data for ^{60}Co γ-rays up to 1 Gy in order to know the effectivity in dose estimations.

Materials and Methods

Cell culture

Peripheral blood samples from 4 healthy volunteers aged between 18 years and 33 years with no history of exposure to mutagenic agents including radiation were obtained by venipuncture using a 21-gauge syringe. In all cases, peripheral blood lymphocytes were separated from whole blood on Fico-Hypaque gradients, washed twice in Hank's balanced salt solution and resuspended in RPMI 1640 (GIBCO, Grand Island, NY) containing Hepes buffer, 15% heat inactivated fetal calf serum, L-glutamine and antibiotics. The lymphocytes were cultured in multi-well tissue culture plates (Corning, No. 25820, NY) at a concentration of 5×10^5 cells/m*l*. An optimum concentration of phytoheamagglutinin (PHA, 5 μg/m*l*, Sigma, St. Louis, Mo) was used to stimulate the lymphocytes to transform and divide in culture. The cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2.

Irradiation condition

One sample served as a control for determining the spontaneous MN frequency. For the elaboration of the dose-effect curve, blood samples were irradiated with 0.25, 0.5, 0.75 and 1 Gy with ^{60}Co γ-rays (Theratron-780 teletherapy unit). The dose rate was 211 cGy/min. The doses were measured with a Capintec PR-06C farmer type chamber and a Capintec 192 electrometer (Capintec, U.S.A.).

Cytokinesis-block methods

Cyt-B (Aldrich Chemical Co., West Saint Paul) was made up as a stock solution in dimethylsulphoxide at a concentration of 2 mg/m*l*, divided into small portions and stored at -70°C. The stocked solution of Cyt-B was thawed, diluted in medium and added 44 h after commencement of the culture at a concentration of 3.0 μg/m*l*. After an incubation period of 72 h, the cells were collected by centrifugation and resuspended in a mixture of methanol: glacial acetic acid(3:1). The fixed cells were transferred to a slide, air-dried and stained with 10% Giemsa for 10 min.

Scoring of micronuclei and data analysis

The MN was scored in 1,000 binucleated CB cells using a 400x magnification. All analyses were performed using a Graph PAD in Plot computer program (GPIP, Graph PAD Software Inc., San Diego) and Excel program.

Results

Induction kinetics of MN in the human peripheral lymphocyte after radiation exposure

A preliminary investigation was done to determine the optimum concentration of Cyt-B for accumulating CB cells. Lymphocytes were exposed to varying concentration of Cyt-B. The optimum Cyt-B concentration appeared to be 3.0 μg/m*l* and this concentration was used throughout the experiments. To find dose-response relationship after γ-rays, the numbers of MN were counted by light microscope (LM). The number of induced MN was obtained by subtraction of the number of cells scored as MN in the control samples from the total number of those cells in the irradiated samples. The morphological findings of the irradiated groups were typical CB micronucleus in lymphocytes, as shown in Fig 1.

The average numbers of MN induced by γ-rays, obtained by pooling the LM data of 4 subjects, are presented as a function of radiation dose and the error bars represent standard deviations within the studied

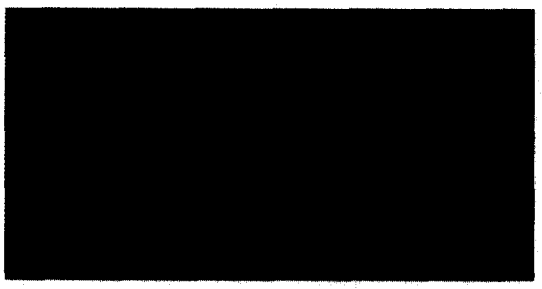

Fig 1. Micrographs of typical cytokinesis-blocked micronuclei in peripheral lymphocytes with Giemsa stain after irradiation with ^{60}Co γ - rays.

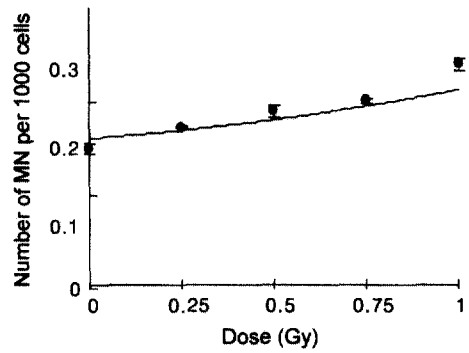

Fig 2. Dose-response curve of micronuclei in binucleated human lymphocytes after γ - rays irradiation.

population. The number of MN increased with both times after irradiation and sizes of dose(Table 1). The dose-response curves presented in Fig 2 indicate that the MN was more sensitive than that of expected process in low dose radiation. The spontaneous MN frequency in lymphocytes of the unirradiated groups showed no significant difference between individuals. The baseline number of MN per cell in unirradiated group was low, being 0.02± 0.0009 (Fig 2).

Dose-response relationship

To evaluate the dose-response curves, the number of MN per cell was examined at the different doses, and the dose-response curve of CB micronucleus was obtained by fitting the linear-quadratic model $y = a+bD+cD^2$, where y is the yield of MN/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 $Y = (0.02\pm 0.0009) + (0.282\pm 0.342)\ D + (0.242\pm 0.269)\ D^2$ ($r^2 = 0.970$) after γ - rays (Fig 2). There was a significant relationship between the frequency of MN and dose. The dose-response curves were linear-quadratic. These data show trends towards increasing MN numbers with increasing

Table 1. Micronucleus frequencies in human cytokinesis-blocked peripheral blood lymphocytes following irradiation.

Donor	Dose (cGy)	No. of cells scored	Micronucleus distribution per cell				Average number of micronucleus/cell
			1	2	3	4	
A	0	1000	11	1			0.013
	25	1000	37				0.019
	50	1000	40				0.040
	75	1000	38	3			0.044
	100	1000	54	2			0.058
B	0	1000	25	2			0.029
	25	1000	33	1			0.030
	50	1000	32	3			0.038
	75	1000	49	2			0.053
	100	1000	56	2			0.069
C	0	1000	11				0.011
	25	1000	25				0.020
	50	1000	29	1			0.031
	75	1000	38	2			0.042
	100	1000	71	3			0.077
D	0	1000	25	1			0.027
	25	1000	38	1			0.029
	50	1000	43	1			0.045
	75	1000	56	1			0.058
	100	1000	71	1			0.073

dose, a plateau being above some higher doses was observed.

Discussion

It is important to develop simple and reliable techniques for evaluating radiation-induced genetic alteration of victims after accidental exposure in case of radiation accident. Until now, there has mainly been relied in the past 20 years on the measurement of chromosome aberrations, which has provided valuable data on the different types of unstable and stable aberrations that can be induced following *in vitro* and *in vivo* exposure[1,8,12]. However, it is difficult to predict the absorbed dose by this indicator at low dose range. As an alternative quantifiable indicator, the CB method is a sensitive end point to estimate the absorbed dose although the enumeration of micronuclei numbers in lymphocytes depends on the proportion of cells that have responded to the mitogen, the proportion of the responding cells that have divided, and the fate of micronuclei in cells which have divided more than once. These factors may vary greatly both between different individuals and technical factors within some individual. At present, therefore, there is no biological indicator that can be used for monitering dose limits of occupational exposure except MN assay. The indicator can definitely be found in the field of physical dosimetry. In radiation accidents involving higher doses, several indicators are available, but not in low doses[12,13]. One of the most prominent perquisites of a biological indicator used in dose estimation is its ability to estimate radiation dose for many people within a short time. Most such studies are based on the analysis of conventional chromosome aberration. The MN assay using the CB method is discussed as a simpler cytogenetic dosimeter, a less expensive and less time-consuming alternative to the traditional scoring of dicentric chromosomes[8,11-15]. Difficulties exist for assessing radiation doses of past exposures because of the temporal decline of cells containing unstable forms. A very attractive method as an alternative means for retrospective biodosimetry is fluorescence in-situ hybridization(FISH) of the chromosome painting such as symmetrical translocations and insertions[16].

In the present study, our results showed more reproducible, dose-related and quantifiable in low dose range than the other scoring system based on visual observation. With this approach it would be possible to detect the effects of doses in case when acute whole-body exposure has occurred and the screening of many victims is necessary because this indicator can be measured easily and seem to be one of the most sensitive radiobiological endpoints in low dose range. After exposure to low dose radiation, the linear-quadratic model is most frequently used to describe the dose-response relationship for micronucleus induction in peripheral lymphocytes with other reports[17-19]. As micronuclei are derived mainly from acentric fragments after radiation exposure[20], one should expect a dose-response relationship with a marked linear component. Micronuclei, however, are not only produced by this one-track mechanism, but also by two-track actions, which become more important at higher doses of low linear energy transfer(LET) radiation. Thus, the inclusion of a quadratic term starting about 1 Gy is both biologically and statistically justifiable[17,21].

In contrast to the effect of higher dose radiation in low and high LET radiation on micronucleus induction, the effect in low dose range is not well documented. After high dose exposure to γ-rays and fast neutrons, we found a linear shape of dose-response relationship in fast neutron and linear-quadratic in γ-rays up to 8 Gy, suggesting a predominance of one-track events in high LET radiation, but two-track in low LET radiation. Exceeding 3 Gy, a tendency to saturation was indicated in our earlier experiments, which is also reflected by a significantly negative b-value derived from fitting to the linear-quadratic model. Such a linear-quadratic in the dose response after low dose exposure was described not only for lymphocytes, but also for hepatocytes. The indication of a saturated MN induction after exposure can be attributed to different phenomena: at higher doses, a reduction of cell proliferation of highly damaged cells occurs and less binucleated cells are formed. Although a sufficient number of binucleated lymphocytes has been scored, one can argue that particularly cells with multiple aberrations have already died before or during the first karyokinesis. Such heavily damaged cells escape from scoring as micronucleated binucleates. Furthermore, the majority of radiation-induced micronuclei originate from acentric fragments. Since with high LET radiations substantially higher numbers of acentrics will be induced than with low LET radiations, many of the MN scored can be assumed to contain more acentric fragment at higher doses than at low doses[21-26]. Here, our data present that MN frequencies increased with increasing radiation doses in statistical distribution.

In conclusion, our results reveal a clear sensitivity of the MN dosimeter at low dose range regardless of radiation qualities. Since micronuclei in CB cell have the potential to complement metaphase analysis of chromosomes for estimating chromosome damage in human lymphocytes, it may be a simple and reliable biological dosimetry for dose estimation at low dose range in the accidental exposure.

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저선량의 감마선에 피폭된 사람 말초 임파구의 미소핵을 이용한 방사선 생물학적 피폭선량 측정법 연구

김 태 환

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(2001년 2월 12일 게재승인)

국문초록 : 불의의 방사선 피폭 환자의 체내 방사선 피폭선량의 예측을 위한 방사선 생물학적 선량 측정 개발의 일환으로 저선량 피폭환자의 체내 피폭선량 측정 지표로서의 미소핵 분석법 이용 가능성을 평가하기 위하여 코발트-60 감마선을 0.25 Gy에서 1 G의 선량을 인체 말초 혈액에 조사한 후 임파구내 미소핵의 수적 변화를 형태학적으로 관찰하였다. 저선량에 피폭된 임파구에서 미소핵이 관찰되었으며, 선량에 따른 수적인 변화도 나타났다. 저선량 피폭에 대한 미소핵의 수적 변화에 대한 선량-반응 곡선은 $Y = (0.02 \pm 0.0009) + (0.033 \pm 0.010) D + (0.012 \pm 0.012) D^2$의 식을 얻었으며, linear quadratic model 이였다. 이상의 결과에서 미소핵의 발생 빈도와 피폭 선량간에 유의한 효과가 있는 것으로 확인되었다. 그리고 정상대조군에서는 세포당 0.02 ± 0.0009개가 관찰되었다. 따라서 말초 임파구를 이용한 미소핵 분석법은 저선량 피폭환자의 체내 피폭선량 측정은 물론 방사선 방호제의 검색 및 방사선 민감도 검사를 위한 방사선 생물학적 지표로 이용 가능하며, 특히 이 방법은 간편하고 정확하며 재현성이 있는 방법으로 불의의 방사선 피폭 사고시 체내 피폭선량을 예측하는 좋은 지표로 사용되어질 수 있을 것으로 사료됨.

Key words : micronucles, peripheral lymphocyte, low dose radiation, biological dosimetry.