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RESEARCH ARTICLE

Assessment of Cytogenetic Damages on Human Peripheral Lymphocytes Following Gamma Rays Local Cutaneous Exposures

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ABSTRACT

The purpose of this paper is to establish the cytogenetic analyses of human peripheral blood samples caused by simulation of partial-body exposures. Either accidental or occupational partial-body exposure to ionizing radiation poses significant health hazards that are indicated by induction of chromosome aberrations (CA). The percentages of mixtures of blood samples irradiated *in vitro* with 2 Gy of gamma rays were 10, 25, 50, 75 and 100.0%. Lymphocytes were cultured for 48 hr, harvested with standard procedures and then first-division metaphase cells were analyzed. It showed that the frequencies of unstable CA depend on the proportion of the irradiated blood. All frequencies of the observed CA was lower than that of predicted or calculated from 100% exposed blood, except in one case, indicating a phenomenon of "dilution" of the un-irradiated into irradiated lymphocytes that may take place a bystander effects. Our data showed that the quantification of CA in human peripheral blood lymphocytes may be an important tool of dose assessment for partial-body exposure to ionizing radiation.

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INTRODUCTION

Scoring of unstable CA including dicentrics, rings and fragments in human peripheral lymphocytes have been extensively studied for estimating individual dose to ionizing radiation (Liu *et al.,* 2010; Lee *et al.,* 2012; Romm *et al.,* 2011). Dose estimate on the basis of an *in vitro* calibration curve is straightforward when the whole-body irradiation is homogeneous (Vaurijoux *et al*., 2012). Radiobiological effects in human peripheral bloods, however, can be significantly different when the partial-body of patients was irradiated (Prasanna *et al.,* 2010), where some problems can arise to evaluate the irradiated fraction of body. This information also plays an important role in medical treatment of victims after radiation accident (Vaurijoux *et al.,* 2012). Several *in vivo* studies on partial-body exposures have been conducted with cancer patients to optimum standardized curves for biodosimetry (Agrawala *et al.,* 2010) while the *in vitro* studies reported on CA induced by gamma rays for simulating local cutaneous exposure by mixing irradiated and normal control blood samples (Vaurijoux *et al.,* 2012; Herodin *et al.,* 2012). It was shown that the in vitro dose–effect relationship is comparable to that of *in vivo* system (Romm *et al.,* 2011). After local cutaneous exposure, peripheral blood samples contain a mixture of irradiated and normal control lymphocytes, which render the evaluation of the overall aberration frequencies more difficult. Irradiated lymphocytes are disadvantaged more in terms of delay of their division or interphase death than normal control lymphocytes (Heimers *et al*., 2006).

Therefore, although the radiobiological effect is species-specific, conventional biodosimetry after local cutaneous exposure can lead to an incorrect estimation of individual dose due to the loss of radiation-induced injured cells in culture for cell division (Pinto *et al.,* 2010). In *in vitro* experiments, this incorrect estimation of radiobiological response was dependent on both the absorbed dose of radiation and the proportion of irradiated and normal control lymphocytes. The distribution of CA among peripheral blood cells can also provide the important data to assess the absorbed dose of victims

regarding to the heterogeneity of exposure that produces an over-dispersed distribution of dicentrics. The mixed blood culture technique, which is trying to simulate local cutaneous irradiation of victims, has been shown to reflect hypothetical situation of the *in vivo* exposures. Interpreting CA data in local cutaneous exposure situations is one of the major problems of cytogenetic dosimetry (Heimers *et al.*, 2006).

In this experiment, peripheral blood samples were irradiated with 2.0Gy of gamma rays for simulation of local cutaneous exposure by mixing irradiated and normal control blood with the proportions of 10 to 100%.

MATERIALS AND METHODS

Blood collection: Peripheral lymphocytes were collected in sterile heparinized vacutainers (Becton Dickinson) from 4 healthy volunteers, one female and 3 male aged between 30 and 47 years old. No medicines or drugs were taken by all volunteers for at least six months prior to sampling and they were free from any diseases for more than 12 months before sampling. They had no recorded over-exposure to radiation in their personnel documents.

Irradiation protocol: All experiment groups were irradiated by γ-rays with a source of Gamma-cell 3000 Elam (Nordion International, KIRAMS, Korea). The dose rate was 3.16 Gy/min. The radiation exposure field was 30 mm in diameter, and all samples were irradiated in air at room temperature. To simulate local cutaneous exposures, the calculated volumes of irradiated blood were mixed with appropriate amounts of normal control blood from the same volunteers to obtain the calculated rates of irradiated blood. Each percentage of mixtures of blood samples irradiated *in vitro* with 2.0 Gy of gamma rays was 10, 25, 50, 75 and 100.0%. Blood with no exposure served as sham control.

Cell culture: CA analysis was according the standard protocol given by the guideline of International atomic energy agency (IAEA) is required with slight modifications. Two milliliters of the mixed blood samples were cultured for 48h in a humidified incubator containing 5% CO_2 at 37^{o}C. Cell culture was carried in 8.0 ml of RPMI-1640 supplemented with 10% heat inactivated fetal bovine serum and 1% streptomycin/ penicillin. The components of culture medium were obtained from Gibco except phytohemagglutinin (PHA) and Colcemid (PHA, Gibco BRL, Grand Island, NY). And 300 µl of PHA was added to stimulate cell division. To block the mitotic process of cells at metaphase stage, Colcemid was added at a final concentration of 0.1 mg/ml in the last 4 h of culturing. Cells were then harvested and centrifuged for 10 minutes at 1,500 rpm and resuspended in 8 ml of 75mM KCl (pre-warmed to 37^{o}C) for 20 minutes. At this stage, 2 ml cold Carnoys fixative was added into test tube and the fixative step was repeated 3 times. The yield of cells at metaphase stage was stored in freezer at least one night until the preparation of slide was made.

Scoring the metaphases: Two slides per each sample were prepared, encoded, and then stained with 10% Giemsa (Merck). The frequencies of CA were observed under a light microscope with magnification of x1000 connected to Olympus CCD Camera System. The cells were considered as aberrant if it has one or more unstable CA such as dicentrics and rings. Scoring of CA was done by a single scorer in complete metaphase with more than 45 centromers only as per the scoring criteria described (Pinto *et al*., 2010). Each sample was scored at least 800 metaphase cells of first division. In normal control samples, 1,000-1,200 metaphase cells were analyzed per donor. Tricentrics and tetracentrics were considered as two and three dicentric equivalents, respectively.

Data analysis: For statistical analysis of all experiment samples, the significance of all data 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

The frequencies of CA in all samples observed after simulation of partial-body is shown in Table 1. There are slight variations in the frequencies of CA for each sample. The frequencies of dicentrics were dependent on the proportion of irradiated blood with a higher proportion exhibiting higher frequencies of dicentrics. In the case of 25% proportion of irradiated blood, a much lower frequencies of CA were seen in subject No. 2 than others, while in the case of 50% proportion of irradiated blood, the frequencies of CA were quite similar in all groups. Very high frequencies of CA in case of 100% proportion were observed in female subject. The total frequencies of CA in all groups were presented in Table 2. The observed yields of CA were below the expected yields calculated from 100% proportion except for subject No. 2 of 10% irradiated blood group at a cultivation time of 48 h after radiation exposure conducted in this experiment.

In this experiment, two other types of CA were also observed. These were terminal translocation that was generally the irradiated portion-dependent, and chromatid breaks/gaps that was no relationship with the portion of irradiated blood, thus the dose of radiation.

As seen in Table 2, totally 17,200 metaphase cells were analyzed in blood samples from four volunteers. In these samples, the results showed significantly high frequencies of CA in group of higher proportion of irradiated blood, providing evidence of radiation-induced CA. These frequencies of CA showed individual variation and the frequencies of dicentrics and rings per cell ranged from 0.0034 in control group to 0.4238 in 100% irradiated group. There were no significant differences in the frequencies of CA among all groups. The number of cells containing more than one dicentric was dependent on the proportion of irradiated cells. Five dicentrics per metaphase cell was observed in as low as 25% of irradiated portion in one as much as the same results were found in 100% irradiated blood of two groups. Several types of CA were observed simultaneously including tricentric and two dicentrics in one metaphase cell of one 100% irradiated group is shown in Fig. 1.

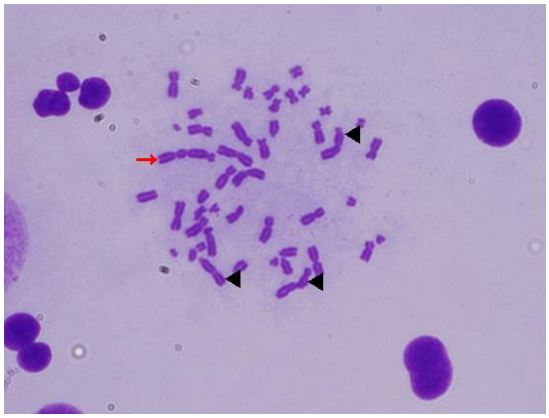

Fig. 1: One tricentric (→) and three dicentrics (◂) chromosomes observed in 100% proportion of 2Gy irradiated blood obtained from one volunteer.

Table 1: Frequencies of chromosome aberrations induced by gamma rays of local cutaneous exposure simulation with 2Gy in four blood samples of healthy adult volunteers

Volunteer no. Frequency of Gender, age	Percentage of Irradiated blood (calculated) *	Total cells counted dicentrics or rings	No. cell with dicentrics and rings	Percentage of cell with dicentrics and rings
1 M/42y	0.0	1200	5	0.0042
	10.0	800	28 (29.2)	0.035
	25.0	800	59 (73)	0.059
	50.0	800	117 (146)	0.117
	100.0	800	292	0.365
2 M/47y	0.0	1200	2	0.0017
	10.0	800	37 (25.7)	0.046
	25.0	800	33 (64.3)	0.041
	50.0	800	97 (128.5)	0.121
	100.0	800	257	0.321
3 F/43y	0.0	1000	4	0.004
	10.0	800	38 (45.5)	0.048
	25.0	800	63 (113.8)	0.079
	50.0	800	177 (227.5)	0.221
	100.0	800	455	0.569
4 M/30y	0.0	1000	5	0.005
	10.0	800	28 (29.2)	0.039
	25.0	800	59 (73)	0.084
	50.0	800	117 (146)	0.230
	100.0	800	292	0.440

DISCUSSION

Generally the analysis of CA is confined to cells at the first post-irradiation mitosis. Due to radiation-induced cell cycle derangements and mitotic delay, it is suggested to extend longer culture time for the expression of heavily damaged cells that reach mitosis later than undamaged or slightly damaged cells. In fact, for sparsely ionizing radiation which has only a slight effect on the time of cell cycle, a few increase of aberration yield with time has been reported (Nasonova *et al.,* 2000; Nasonova *et al.,* 1998). In our experiment, the culture time was not extended due to low dose of irradiation applied such as 2Gy, but our protocol was similar with other data on simulation of partial-body exposure although radiation sensitivity is different (Herodin *et al.,* 2012). The yields of CA in our experiment were included abnormal cells that may affect the extent of their first mitosis or inter-phase death in order to estimate correctly the absorbed dose of victims. Some studies reported that longer culture time for biodosimetry is recommended to avoid an incomplete count of CA frequencies and an under-assessment of radiation exposure (Liu *et al.,* 2010).

In our results, the frequencies of CA were increased with culture time. This is also supported by the findings in other data of victims involving high dose of partial-body exposure below 30Gy where only three dicentrics and rings per 500 metaphases were counted in 48 h cultures (Lloyd *et al.,* 1996). Therefore, a considerable prolongation of culture time with an increase of metaphasic lymphocyte is to be recommended for the biodosimetry of local cutaneous exposure regardless of human species. Recently, the frequencies of unstable CA for simulation of partial-body exposures with 200 kV X-rays and 2.1 MeV neutrons by mixing irradiated and normal control blood in proportions of 25, 50, and 75% was reported from 48 hour to 72 hour on culture times (Heimers *et al.,* 2006). We observed that strong mitotic delay occurs in irradiated lymphocytes. Also we found that the quantitative extent of unstable CA depends on the number of irradiated blood and the dose. Culture time in our experiment was only at 48 h, but the frequencies of CA were observed increasingly with the manner of dose-dependent at all groups. In the case of 25% proportion, the lower frequencies of CA were seen in subject No. 2. This may be related to individual variation of resistance to radiation. Very high frequencies of CA in case of 100% proportions were observed in female subject, possibly due to the specific characteristic of gender. The observed yields of CA were below the expected yields calculated from 100% proportion at a culture time of 48 h conducted in this experiment. This result is in agreement with the reported study regardless of different species (Herodin *et al.,* 2012). With the same low-LET radiation we compared to other results of experiment conducted by Heimers (Heimers *et al.,* 2006) and Herodin (Herodin *et al.,* 2012) who also used X-rays, the frequencies of dicentrics plus rings in simulation of partial-body exposures were comparable. This comparison is presented in Table 3, which is available only for 25-100% proportion of irradiated blood. In their experiment, dose rate used by Barquinero (Herodin *et al.,* 2012) was 0.2695 Gy/min, while dose rate of our experiment and Heimers (Heimers *et al.,* 2006) was 3.16 Gy/min and 0.52 Gy/min, respectively. Our results were well agreed with the data of experiment by the other scientists (Herodin *et al.,* 2012; Knops *et al.,* 2012) who reported that the decrease of CA frequencies is dominantly influenced by the proportion of the irradiated blood. This can lead to an underestimation of the exposed body part and the total dose. The entry phenomenon of mitosis of damaged lymphocytes is mainly influenced by the exposed doses, irradiated cell volumes, and the selective advantage of normal control lymphocytes. This result can be explained by a dilution effect of the highly exposed lymphocytes which includes a high proportion of aberration-carrying cells with normal control intact lymphocytes as the same of our data. It has been clearly observed that dicentrics were found to have no accompanying acentric fragment, because the fragment possibly joined to other chromosomes as a terminal translocation in some finding. Terminal translocation was found to be increased with the absorbed dose of peoples suspected of being overexposed to ionizing radiation (Sorokine-Durm *et al.,* 1997). Because coincidences of

Table 2: The total frequencies of chromosomal aberrations induced by gamma rays of local cutaneous exposure simulation with 2Gy in four blood samples of healthy adult volunteers

% of irradiated blood	No. cells counted	No. cell with D or R*	No. of chromosomes containing			No. of lymphocyte containing dicentrics and or rings						Frequency of D and R
			D	R	*F*	0	1	2	3	4	5	
0.0	4,400	15	14	1	7	4,385	14	1	0	0	0	0.0034
10.0	3,200	134	121	13	36	3,066	122	9	3	0	0	0.0369
25.0	3,200	222	208	14	120	2,968	197	21	2	1	1	0.0644
50.0	3,200	575	533	42	315	2,625	473	84	18	0	0	0.1797
100.0	3,200	1,356	1,245	111	755	1,844	1,114	177	36	6	2	0.4238

* D = dicentrics, R = rings, F = fragment (not including fragment that accompany dicentrics and rings induction).

Table 3: The comparison of frequency of dicentrics plus rings in lymphocyte cells induced by radiation in simulation of local cutaneous exposure of this experiment and two others

Percentage of Irradiated blood	Frequency of dicentric and rings obtained in Our experiments	Heimers et al.[13]	Barquinero et al.[14]
25.0	0.064	0.086	0.041
50.0	0.179	0.157	0.131
100.0	0.423	0.424	0.280

translocations and dicentrics in the same cell will occur, particularly in a high dose, there will be some loss of translocations when the unstable cells divide, but will not cause a reduction in the yield of translocations. By contrast, the distributions are not independent in local cutaneous exposure with high dose because translocations and dicentrics are confined to the proportion of irradiated cells, but dicentrics decline with time.

Partial-body exposure will result in a heterogeneous proportion of lymphocytes, but the resultant dicentric yield will produce more realistic estimation of the absorbed dose. In this relation, dicentrics are currently the most sensitive and reliable indicator that can be used for biodosimetry. These also can be used as complementary methods to physical dosimetry. Accordingly, this biodosimetric technique is currently being developed for evaluating radiobiological effect (Lee *et al.,* 2012). In addition, significant radiation-induced changes in the frequency of CA have been detected at very low doses (Agrawala *et al*., 2010). Dicentrics are unstable CA because they are not transmissible forms and they are eliminated with time and its mean half-life is estimated to be about 130 days (Natarajan *et al.,* 1998).

As stated above, the estimation of partial-body exposure is complicated by mitotic selection induced in radiosensitive lymphocytes after local cutaneous exposure with ionizing radiation (Yao *et al.,* 2013). In our experiment, we measured CA in peripheral blood lymphocytes by using the conventional technique. The yields of CA were dependent on the size of irradiated blood. This result is expected and can be explained in the mixed culture by a dilution effect of the highly exposed lymphocytes which includes a high proportion of aberration-carrying cells with normal control intact cells.

Conclusion: Finally, dose estimates are crucial for risk assessment as well as for clinical treatment of victims. The results presented in our experiment concluded that CA is a reliable method for investigating dose assessment of local cutaneous irradiation. Thus, this experiment could not only help in better dose estimation of local cutaneous exposure with ionizing radiation, without the difference and scarcity of human data on dispersion analysis of cytogenetic damage depending on species, sex, gender, life style and so on, but also constitutes a preliminary step in the process of defining the possibilities of this cytogenetic biomarker to cover the uncertainty of radiation effect after local cutaneous irradiation.

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