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Frequencies of Micronuclei in Peripheral Lymphocytes in Korean Populations after Chronic Low-dose Radiation Exposure

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Abstract

The purpose of this study was to estimate predictive markers of intrinsic radiosensitivity in individuals who were exposed to occupational or environmental radiation. Throughout this process, the actual biohazard risks and base-line chromosome damage were evaluated in human population. Further studies were carried out to provide evidence for the existence of individual variations in age-dependent responses through micronuclei (MN) assay.

Spontaneous frequencies not only vary greatly between individuals, but also working or living areas. It was shown that the increased level of spontaneous cell with MN was observed with increasing age. The relationship between radiosensitivity and the increased spontaneous level of MN may be in an inverse proportion. Ionizing radiation may be targeted mutagenic effects at the usual exposures of background levels that populations were exposed. Age and gender are the most important demographic variables in determining the MN index with frequencies in females, which were greater than those in males. The main life-style factors influencing the MN index in subjects were correlated significantly and positively with smoke. The results showed that an indicator of the genetic damaged rate in MN index in human populations significantly correlated with age, sex and life-style factors. So far, it is evident that with regard to the application of MN assay all future studies have to take into account the influence of age, gender, and life-style.

In Conclusion, using micronuclei assay technique a large population can be easily monitored. This study illustrated that the MN assay may provide a high potential to ensure appropriate quality control and standard documentation protocol that can be used to monitor a large population exposed to radiation epidemiologically.

Key words : Micronuclei, Lymphocyte, Radiation biological dosimetry, Chronic radiation exposure, Epidemiological studies.

Introduction

Since radiation effects are misunderstood and feared by the public, the hazard of various forms of it can be anxiety provoking. Human and animal populations are at risk from various forms of biohazardous exposure to environmental agents. For example, the various forms of radiations such as ionizing radiation, ultrasound and microwaves have the potential for producing biological effects, which varies considerably with radiation qualities and dose. In evaluating the actual biohazard risks, the cytokinesis-blocked micronuclei (MN) assay has been adopted by numerous researchers as a means for rapidly assessing base-line chromosome damage in human population as the sensitive and reliable method. This biomarker for measuring the cytogenetic damage would be of great value in monitoring genetic risk in human population such as hospital workers, nuclear power plant workers, residents around radiation related facilities and inhabitants in high background areas. In circumstances that the physical dosimetry is unavailable or unreliable, the level of radiation-induced genetic damage can estimate the extent of exposure. At present, this is usually determined the frequencies of dicentric chromosomes in the peripheral blood lymphocytes. Although this is a sensitive method for dose estimation, it is laborious and takes extensive experience to estimate correctly. Unfortunately, without automation of this method, its use and scope for population screening is limited. Especially, the chromosome aberration has various confounding factors in assessing the irradiated dose in cases of very low-dose exposure. Therefore, we need an alternative cytogenetic dosimetry to estimate the absorbed dose of victims after low-dose exposure such as hospital workers,

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workers of radiation related facilities and inhabitants in high background areas[1-7].

An alternative indicator as a reliable biomarker for monitoring chromosome damage is MN presence in the peripheral lymphocytes. Micronuclei arise from acentric chromosomes fragments that are not included in the daughter nuclei of a dividing cell because the fragment does not successfully engage with the spindle. Whole chromosomes damaged at the centromeric region or damaged to kinothchore or spindle proteins may also result in unsuccessful attachment of a whole chromosome with the spindle resulting in a lagging chromosome at anaphase which subsequently increases MN. Thus the MN test is a reliable measure of both chromosome loss and breakage which make it unique compared to other cytogenetic tests. With this key performance characteristic, the cytogenesis-blocked (CB) MN assay is now at a stage of acceptance and development, both conceptually and methodologically to be used to provide a more in-depth study of chronic low-dose radiation exposure and damage. More data is still required for the process of its validation as a biomarker that could predict specific health outcomes such as rate of ageing, radiosensitivity and cancer. It is also important to establish standardized protocols that would enable more reliable data comparison between laboratories worldwide, as this could help identify different life style factors that impact on base-line genetic damage rates and help to reliably define acceptable and 'normal' DNA damage rates in human populations. However, few studies have been performed to develop the biomarker in measuring chromosome damage of human populations with chronic low-dose exposure to ionizing radiation, or even until recently, had not developed any recognizable and reliable techniques for measuring chronic low-dose exposure below background level[8-11].

Accordingly, to determine the usefulness of MN assay as a reliable biomarker for monitoring chromosome damage of peripheral lymphocytes in human populations following chronic low-dose exposure to ionizing radiation below background levels, the present study using the MN assay was performed to monitor the intrinsic radiosensitivity and chromosome damage of human populations by environmental radiation exposure.

Materials and Methods

Cell culture

Peripheral blood samples from 81 healthy volunteers aged between 18 years and 70 years were obtained by venipuncture using a 21-gauge syringe. Volunteers were taken from different age, gender and areas such as those who work at the radiation power plant, as well as those individuals who live in the area. 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/ml. An optimum concentration of phytoheamagglutinin (PHA, 5μg/ml, Sigma, St. Louis, Mo) was used to stimulate the lymphocytes to transform and divide in culture. The cells were cultured at 37℃ in a humidified atmosphere containing 5% CO_2.

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/ml, divided into small portions and stored at -70℃. Then, the stocked solution of Cyt-B was thawed, diluted in medium and added 44 hrs after commencement of the culture at a concentration of 3.0μg/ml. After an incubation period of 72 hrs, 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 acridine orange.

Scoring of micronuclei and data analysis

The MN was scored in 1000 binucleated CB cells using a 400× magnification after staining. 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 peripheral lymphocytes in human populations

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 3.0μg/ml and this concentration was used throughout the experiments. The numbers of MN were counted by light microscope (LM). The number of observed 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 separated groups. The morphological findings of observed MN were typical in lymphocytes. As shown in Fig. 1, the average numbers of MN induced, obtained by pooling the LM data of subjects, are presented as a mean and the error bars represent standard deviations within the studied population.

In epidemiological studies of base line in each population group showed the existence of individual variations in age-dependent responses to radiation. The spontaneous MN frequencies varied greatly not only between individuals, but also between the group of working and living in the radiation power plant. The spontaneous MN frequency in lymphocytes of the control groups showed no significant difference between individuals. It was shown that the

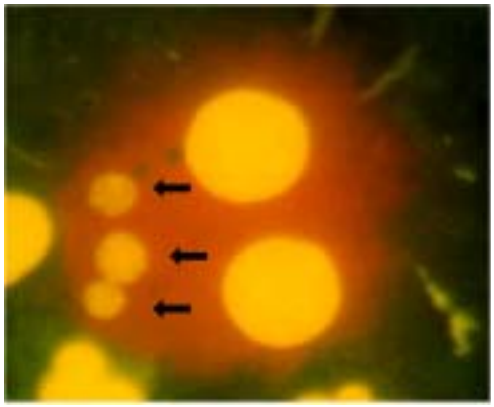

Fig. 1. Photomicrograph of cytokinesis-blocked lymphocyte containing micronuclei(arrows). Acridine Orange stain, × 1000

increased level of spontaneous cells with MN was observed with increasing age. The relationship between radiosensitivity and the increased spontaneous level of MN may be in an inverse proportion. Age and gender are the most important demographic variables impacting on MN index with MN frequencies in females being greater than those in males depending on the age group. For both sexes, MN frequency was significantly and positively correlated with age. The main life-style factors influencing the MN index in subjects are correlated significantly and positively with smoke. The results showed that an indicator of the genetic damaged rate in MN index in human populations significantly correlated age, sex and life-style factors (Table 1, 2).

Table 1. Frequencies of Micronucleus in human peripheral lymphocytes according to age and sex group for Korean baseline study.

Age groups		Females(n)	Males(n)
20≤29	Average No. of MNs	5.85±4(27)	9.33±2.94(3)
30≤39	Average No. of MNs	5.87±3(15)	-
40≤49	Average No. of MNs	9.11±5.41(9)	10.67±3.89(3)
50≤59	Average No. of MNs	18.67±8.19(3)	14.75±5.74(4)
60≤	Average No. of MNs	21.22±9.75(6)	13±5.73(11)

Table 2. The effect of smoker on the frequencies of micronucleus in human peripheral lymphocytes.

Variable	Average Number of MN Frequencies
smoker	14.89±4.96(14)
nonsmoker	7.95±5.94(67)

Discussion

It is important to develop a simple and reliable biomarker for assessing radiation-induced genetic alteration of human populations. In the past 20 years, the measurement of dicentric chromosome has been the only source relied on to provide 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]. To date, there has not been a comprehensive and coordinated international effort to identify the key effects and variables influencing the chromosome damage in lymphocytes of human populations. However, it is difficult to develop reliable and predictive biomarker for measuring radiation-induced chromosome aberration. It is with this in mind that the goals and preliminary data are described below. As an alternative quantifiable biomarker, the MN 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 the cells which have divided more than once. These factors may vary greatly both between different individuals and the technical factors within some groups.

At present, therefore, there are few biomarkers that can be used for monitoring dose limits of occupational exposure. The MN assay could be use as a possible biomarker and indicator. The MN indicator can definitely be found in the field of physical dosimetry[12,13]. One of the most prominent perquisites of a biological indicator used in dose estimation is its ability to estimate radiation doses for many people within a short time. 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 of chromosomes. 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]. Where human judgment is required in the scoring of a cytological end-point for the extent of inter-individuals is one of the most important sources of variation with the MN data as well as with all other methods.

Three experienced experts typically evaluated all data of the inter-group to minimize the impact of inter-individuals variability at the same age and groups. The data obtained from groups of the comparison study show a good correlation between data obtained on each group for mutagenic effects at the usual exposure to the population. When evaluating the actual genetic damage and acquired diseases affecting parents and their offspring, many problems were associated with the risk of induced chronic exposures to environmental agents, but we established dose-response relationship in the vitro model system using many kinds of biomarker such as chromosome aberration, MN, apoptosis induction assay, premature chromosome condensation assay and fluorescence

in-situ hybridization assay in epidemiological studies and experimental studies. The epidemiological studies must demonstrate consistency of genetic damage in human populations by specific biomarker. The result data from the epidemiological studies should be used when designing in vitro experiments.

When the frequency of MN increased depending on age, gender and life style, the radiation exposures to the population were extremely low. There was no data regarding the increase of any genetic effect studies, including congenital malformations and cancer. However, populations exposed to large doses of ionizing radiation have increased incidences of cancer. These populations include the radium dial workers, uranium miners, patients receiving radiation or isotope therapy for various diseases, and the individuals who received the higher exposures in Hiroshima and Nagasaki following the atomic bomb detonation. Few people are aware of the risk of the occurrence of cancer in populations exposed to lower doses of radiation. The immense psychological consequences of high-energy radiation exposure are extremely important and cannot be ignored, when one considers the deleterious effects of radiation. Although there is some overlap with regard to biological effects of ionizing radiation, there are also significant differences. Low LET radiations are mainly stochastic effects, while high LET radiations are mainly deterministic effects. The stochastic genetic risks are lower than the deterministic risks at equivalent exposures. Thus, it is frequently difficult to demonstrate the occurrence of stochastic effects in human populations that have received continuous low-dose exposures to ionizing radiation.

In the evaluation of the actual genetic risks such as chromosome aberrations, we rely on accurate biodosimetry and information obtained in epidemiological and animal studies. The epidemiological studies must demonstrate consistency of genetic findings of damage, and the animal studies should be designed to add to the findings of the epidemiological studies. Most importantly, the findings must not contradict the basic principles of teratology, genetics and biological effects and should be biologically plausible. But frequently, the important basic science principles are ignored in the evaluation process. Yet, genetic basic science principles can be instrumental in refuting or supporting the concern about possible risks.

The problem of emotionality is frequently magnified in the evaluation of radiation risks, as the unschooled are unaware of the marked differences in the biological effects of different forms of radiation. Only the very high-frequency photons of ionizing radiation such as γ-rays, X-rays, α-rays, β-rays and neutron can remove orbital electrons and produce ionization in tissues, resulting in cytotoxicity, chromosome damage and point mutation. Thus, one would expect some biological effect from all forms of radiations. In some instances, these effects are deleterious, but in other instances the effects are reversible or barely perceptible. The actual risk can only be determined by an analysis of many extensive in-depth studies of physical and biological data. In general, one could conclude that there is far more differences in the biological effects of these various forms of radiation. Furthermore, the risks from exposures to environmental radiation exposures vary both quantitatively and qualitatively based on whether the exposures occurred whole or partially. It is important that the significance of dose is not ignored when characterizing the manifestations of exposures to each form of radiation. One cannot evaluate the biological effects and the risks without knowing the type of radiation exposure, its dose rate and the actual exposure. Since ionizing radiation can result in both chromosome damage and point mutation, it is logical to conclude that radiation exposure should increase the risk of genetic diseases such as genetic malformation, hereditary diseases and cancer. The risk, of course, is related to the dose. The genetic and carcinogenic effects of ionizing radiation are considered stochastic phenomena. The characteristics of stochastic phenomena are that although the risk is dose related, theoretically, there is no dose that does not present a risk. Whether the dose-response curve is linear or quadrilinear is a moot point because, at very low doses, the risks from low LET radiation are far below the spontaneous incidence of mutations. Another characteristic of stochastic phenomena is that while the risk increases with dose, the severity of the disease does not. Thus, one cannot distinguish between a patient with leukemia that has occurred spontaneously or after an exposure to a high dose of ionizing radiation. This is because stochastic phenomena are diseases that theoretically arise from the alteration of DNA in a single cell. In regard to the induction of mutations, the greater current risk seems to result from exposure to chemical mutagens in the environment rather than from the populations' exposure to radiation. Thus, The BEIR V committee and geneticists who studied the radiated populations in Japan are convinced that there were radiation-induced mutations. However, the calculated and demonstrated risks are so small that these investigations were unable to demonstrate statistically significant genetic effects, although the population studies were quite large. Ionizing radiation has been demonstrated to be mutagenic in both in vivo animal studies and in vitro systems. Furthermore, radiation can readily produce genetic damage in both males and females if the dose is high enough. The largest study ever undertaken to examine the genetic effects of ionizing radiation occurred after the atomic bomb detonation in Hiroshima and Nagasaki. The offspring of the atomic bomb survivors who were exposed to a single dose of radiation had no measurable increase in induced mutations after exposure to an agent that is a potential mutagenic. Statistical analysis of the incidence of chromosome abnormalities demonstrated no significant increase in the frequency of chromosomal abnormalities in children, neonates and abortuses born to the irradiated parents. A number of laboratories have attempted

to perform a risk assessment of the mutagenic effect of various environmental agents. This is not an easy task because the actual impact of the mutagenic effects is related to dose, time of genetic damage, and time after exposure. Furthermore, the best methodology for determining human risks is to utilize human epidemiological data. The problem with using human data is that the population has been exposed to radiation, even when the exposure is very low. When dealing with low-risk phenomena, one needs large populations to demonstrate an effect. In many human epidemiological studies, the populations are so small that even if there is slight genetic damage, it would not be discerned. Therefore, we continue to study Korean epidemiological survey to estimate human genetic risks according to age, gender, life style, job and so on[17-21].

In estimating the genetic hazards of environmental mutagens there are major problems applying the epidemiological data to biodosimetry in the exposed person. The occupational, medical and population exposures of various forms of ionizing radiation may have the ability to alter DNA without affecting other cellular functions and these toxic effects are deterministic and stochastic effects. Therefore, if genetic effects are to be manifested from low-dose exposure to ionizing radiation through epidemiological and in vitro studies, the risks have to be considerably larger than the risks from non-ionizing radiation or other environmental mutagens.

The data from epidemiological study illustrated that there are developmental methods of improving our abilities to apply MN assay in vitro cellular systems for determining mutagenic risks in human populations. There has been continued interest in the impact of environmental mutagens including ionizing radiation with the construction of many reactors in Korea. However, the animal and human data support the contention that exposures within 0.05Gy range would not expect to increase the incidence of anatomic malformations, growth retardation, mental retardation, or abortion from diagnostic exposure, but not all such epidemiological studies are negative. We have investigated various biomarkers to estimate genetic damage and have not found them to be more sensitive to ionizing radiation than chromosome aberration assay. That does not mean, however, that some other parameters that have not yet been studied would not result in the biological effects from low-level radiation. When evaluating studies dealing with the biological effects of environmental background level radiation, the important principles should guide the analysis of genetic damage in human populations. 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. After exposure to low-dose radiation, the linear-quadratic model is most frequently used to describe the dose-response relationship for micronuclei induction in peripheral lymphocytes with other reports. As micronuclei are derived mainly from acentric fragments after radiation exposure, 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 1Gy is both biologically and statistically justifiable[17, 22-27].

In conclusion, our results reveal a clear sensitivity of the MN at low-dose range below background level. Since micronuclei in CB cell have the potential to complement metaphase analysis of chromosomes for estimating chromosome damage in human lymphocytes below background level, it may be a simple and reliable biomarker for epidemiological studies in occupational workers and residents in high background regions.

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References

1. Brewen JG, Gengozian N. Radiation-induced human chromosome aberrations, human in vitro irradiation compared to in vitro and in vivo irradiation in marmoset leucocytes. *Mutat Res*, 13:383-389, 1971.
2. Szumiel I. Review: ionizing radiation-induced cell death. *Int J Radiat Biol*, 66**:** 329-341, 1994.
3. Clemenger JFP, Scott D. A comparison of chromosome aberration yields in rabbit blood lymphocytes irradiated in vivo and in vitro. *Int J Radiat Biol*, 24:487-491, 1973.
4. Kim TH, Kim SH, Kim JH, et al. Measurement of apoptotic fragments in growing hair follicles following gamma-ray irradiation in mice. *Anticancer Res* 16: 189-192, 1996.
5. Kim SH, Kim TH, Chung IY, et al. Radiation-induced chromosome aberration in human peripheral blood lymphocytes in vitro: RBE study with neutron and ^{60}Co -rays. *Korean J Vet Res*, 17: 21-30, 1992.
6. Sellins KS, Cohen JJ. Gene induction by gamma-irradiation leads to DNA fragmentation in lymphocyte. *J Immunol*, 139: 3199-3206, 1987.
7. Sobels FH. The parallelogram: An indirect approach for the assessment of genetic risks from chemical mutagens. *Progr Mutat Res*, 3**:** 323-327, 1982.
8. Countryman PI, Heddle JA. The production of micronucleus from chromosome aberrations in irradiated cultures of human lymphocytes. *Mutat Res*, 41:321-328, 1976.
9. Fench M, Morley AA. Kinetochore detection in micronuclei: an alternative method for measuring chromosome loss.

Mutagenesis, 4**:** 98-104, 1989.
10. Fench M. The cytokinesis-block micronuclei technique and its application to genotoxicity studies in human populations. *Environ Health Perspect*, 101:101-107, 1993.
11. Cheng TJ, Christiani DC, Xu X, et al. Increased micronuclei frequency in lymphocytes from smokers with lung cancer. *Mutat Res*, 349:43-50, 1996.
12. Savage JRK. A comment on the quantitative relationship between micronuclei and chromosomal aberrations. *Mutat Res*, 207**:** 141-146, 1988.
13. Muller W-U, Sreffer C. Micronucleus assay. In: Obe G, ed. Advances in mutagenesis research. Berlin: Springer. 4:1-133, 1994.
14. Catena C, Conti D, Del Nero A, et al. Interindividual differences in radiation response shown by an in vitro micronucleus assay: effects of 3-amino-benzamide on X-ray treatment. *Int J Radiat Biol*, 62**:** 687-694, 1992.
15. Thierens H, Vral A, De Ridder L. Biological dosimetry using the micronucleus assay for lymphocytes: inter-individual differences in does-response. *Health Phys*, 61**:** 623-630, 1991.
16. Straume T, Lucas JN, Tucker JD, et al. Biodosimetry for a radiation worker using multiple assays. *Health Phys*, 62**:** 122-130, 1992.
17. Ganteberg H-W, Wuttke K, Streffer C, et al. Micronuclei in human lymphocytes irradiated in vitro or in vivo. *Radiat Res*, 128**:** 276-281, 1991.
18. Littlefield LG, Sayer AM, Frome EL. Comparison of dose-response parameters for radiation-induced acentric fragments and micronuclei observed in cytokinesis-arrested lymphocytes. *Mutagenesis*, 4**:** 265-270, 1989.
19. Prosser JS, Moquet JE, Lloyd DC, Et al. Radiation induction of micronuclei in human lymphocytes. *Mutat Res*, 199**:** 37-45, 1988.
20. Neel JV, Schull WJ, Awa AA, et al. The children of parents exposed to atomic bombs: Estimates of the genetic doubling dose of radiation for humans. *Am J Hum Genet*, 46:1053-1072, 1990.
21. Okada S, Hamilton JB, Egami N, et al. A review of thirty-year study of Hiroshima and Nagasaki atomic bomb survivors. *J Radiat Res II*, Suuppl:164, 1975.
22. Evans HJ. Mutation cytogenetics: past, present and future. *Mutat Res*, 204**:** 355-363, 1988.
23. Kim SH, Kim TH, Yoo SY, et al. Frequency of micronuclei in lymphocytes following gamma and fast-neutron irradiations. *Anticancer Res*, 13: 1587-1592, 1993.
24. Bauchinger M, Schmid E, Rimpl G, et al. Chromosome aberrations in human lymphocytes after irradiation with 15.0-MeV neutrons in vitro. I. Dose-response relation and RBE. *Mutat Res*, 27**:** 103-109, 1975.
25. Llyod DC, Purott RJ, Dolphin GW, et al. Chromosome aberrations induced in human lymphocytes by neutron irradiation. *Int J Radiat Biol*, 29**:** 169-182, 1976.
26. Jacobsen L, Mellemgaard L. Anomalies of the eyes in descendants of women irradiated with small X-ray doses during age of fertility. *Acta Ophthalmol*, 46:352, 1988.
27. Vral A, Verhaegen F, Thierens H, et al. Micronuclei induced by fast neutrons versus ^{60}Co gamma-rays in human peripheral blood lymphocytes. *Int J Radiat Biol*, 65: 321-328, 1994.