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業績報告書シリーズ

原爆被爆者のG_0期リンパ球の試験管内線量生存率反応: 高線量群における集団偏向の検査§

Dose Survival of G_0 Lymphocytes Irradiated in Vitro: a Test for a Possible Population Bias in the Cohort of Atomic-bomb Survivors Exposed to High Doses

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要　約

末梢血リンパ球に試験管内でX線を照射し，コロニー法による線量生存率調査を行った。AHS対象者でDS86線量が0.005 Gy未満の117名と，1.5Gy以上の84名についての調査の結果，平均D_{10}値（90%の細胞を殺すX線線量）とその変動係数（CV）は前者で 3.40 Gy（CV = 7.5%），後者で3.34 Gy（CV = 7.8%）であった。両者の間に統計学的な有意差は認められなかった。また，試験管内におけるリンパ球の放射線感受性には，調査対象者のいづれの群においても，また，全員まとめてみても，性別や年齢による影響は認められなかった。したがって，G_0期リンパ球のコロニー法に関するかぎりは，原爆被爆者の高線量被曝群の中から，特に細胞の放射線感受性の高い人が多く亡くなったという証拠は得られなかった。留意すべき点は，ここで観察された細胞の放射線感受性の個人差は，実験間の変動と比較して大きいものではなかったことである。したがって，上記の結果は，リンパ球の放射線感受性に関する個人差が被爆者間で大変少ないことによるものと解釈されるべきものである。

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Technical Report Series

Dose Survival of G_0 Lymphocytes Irradiated in Vitro: a Test for a Possible Population Bias in the Cohort of Atomic-bomb Survivors Exposed to High Doses

Nori Nakamura,[a] Richard Sposto,[b,c] Mitoshi Akiyama[a]

Summary

An in-vitro colony assay was employed for X-ray dose-survival studies of peripheral-blood lymphocytes from 117 Adult Health Study participants with Dosimetry System 1986 doses <0.005 Gy and from 84 participants with doses of ≥1.5 Gy. The mean (coefficient of variation [CV]) D_{10} values (the X-ray dose required to kill 90% of cells) for these two groups were 3.40 Gy (7.5%) and 3.34 Gy (7.8%), respectively. No statistically significant differences in their distributions were detected. In addition, neither sex nor age affected the in-vitro radiosensitivity of lymphocytes for either group or for all subjects combined. Therefore it was concluded that, as far as the G_0-lymphocyte colony assay is concerned, there is no evidence for preferential loss of individuals with higher cellular radiosensitivity among the high-dose atomic bomb survivors. However, it should be noted that the interindividual variations in cellular radiosensitivity were not large compared with the experimental variations. Consequently, the above-mentioned results should be considered due to the small heterogeneity of lymphocyte radiosensitivity among the survivors.

Introduction

Epidemiologic data for atomic bomb (A-bomb) survivors in Hiroshima and Nagasaki are an important source of information for assessing risks of various late effects induced in human populations by exposure to ionizing radiation.[1] However, no extensive survey has yet been carried out to characterize the

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distribution of radiosensitivities among the A-bomb survivors. This is a prerequisite for extrapolation from the experience of A-bomb survivors to other populations: if the A-bomb survivors can be regarded as representative of a normal population in terms of radiosensitivity, then such extrapolations can be made more or less directly; if not, knowledge of the sizes and directions of the deviations from normality may provide the means to adjust the extrapolations.

Studies with mice have shown that each genetically identical inbred strain has its own spectrum of susceptibility to various types of endpoints, such as LD_{50} (the lethal dose for 50% of the population) or tumor induction.[2–5]

Since humans are regarded as genetically heterogeneous to a considerable extent, it is conceivable, a priori, that radiation response also varies among individuals. Indeed, some studies have suggested that human populations are not homogeneous with regard to cellular radiosensitivity and that patients with hereditary diseases who have altered cellular radiosensivity do exist, eg, ataxia telangiectasia (AT) patients.[6–9]

In-vitro studies to evaluate the variation of cellular radiosensitivity in humans required the development of cell culture techniques. Techniques for culturing normal diploid fibroblasts derived from skin have been in use since the 1960s. A serious problem with using this method here at the Radiation Effects Research Foundation (RERF), however, is the difficulty of obtaining skin samples from Adult Health Study (AHS) participants. A better approach is to immortalize easily obtained peripheral-blood lymphocytes (B cells) in vitro with Epstein-Barr virus transformation. However, the cloning efficiency of such cells is usually quite low, and the transformation may result in altered repair capability.[10,11] Therefore, results based upon the B-cell lines involve some ambiguities that preclude making direct interpretations.

The discovery of the T-cell growth factor interleukin-2 (IL-2) now allows us to establish long-term cultures of normal T cells in vitro without the two problems of altered cell characteristics and difficulty in obtaining samples.

Using this (IL-2) culture system, the present study was intended to test for signs of preferential loss of individuals with higher cellular radiosensitivity from the high-dose group of A-bomb survivors.

Materials and Methods

Study cohort

The blood samples were obtained from 201 AHS participants from 1988 to 1990. The composition of the AHS cohort has been described in detail previously.[12] The heavily exposed (high-dose) group comprised persons with DS86 doses ≥1.5 Gy.[13] The control (0 Gy) group comprised persons with DS86 doses <0.005 Gy. Donor age varied from 42 to 86 yr.

Medium and feeder cells

In each well of 96-well microplates (round bottom, Costar #3799, Cambridge, Mass), 2×10^4 fresh lymphocytes, isolated as described below from three laboratory volunteers, and 1.5×10^4 OKIB cells, a B-cell line, were seeded into 0.1 mL of growth medium after exposure to 50 Gy of X-irradiation. The medium was MEM supplemented with 1% nonessential amino acids, 24 mM HEPES, 1%

L-glutamine, 9% fetal calf serum (FCS), 1% pooled human serum of any ABO type, and 0.05% phytohemagglutinin P (PHA-P).[14] The feeder cells were prepared one day before the irradiation experiments.

Lymphocytes

Peripheral blood lymphocytes were collected by glass-bead defibrination and Ficoll-Hypaque separation as described previously.[15] After being washed twice with Earle's balanced salt solution containing 2.5% FCS, 100 U/mL penicillin, and 100 μg/mL streptomycin, the cells were counted after the mononuclear cells were stained using Türk's solution. The cells were diluted with medium devoid of PHA and were distributed to wells containing feeder cells with 0.1 mL of the growth medium supplemented not with PHA, but with 0.2 ng/mL (4 IU/mL) of recombinant IL-2 (provided by Takeda Pharmacy Ltd, Tokyo).[14] Thus, the final concentrations of PHA and recombinant IL-2 were 0.025% and 0.1 ng/mL (2 IU/mL), respectively. For the distribution of feeder cells and test lymphocytes, a 12-channel Titertek dispensor (Flow Laboratories, McLean, Va) was used. The average number of lymphocytes seeded per well was 2 cells for 0 and 1 Gy, 3 cells for 2 Gy, 5 cells for 3 Gy, 20 cells for 4 Gy, and 70 cells for 5 Gy. One plate was used for each dose level.

Immediately after the seeding, the plates were exposed to X-irradiation. Our previous study showed that lymphocytes irradiated from 1 hr before to 4 hr after the addition of PHA did not show a significant difference in radiosensitivity.[14] In the present study, cells were X-irradiated within 1 hr after seeding in medium containing PHA. We consider the dose-survival results to be representative of G_0 lymphocytes.

In most cases, feeder cells were prepared on Monday and the blood samples were obtained on Tuesday morning. Irradiation experiments were completed on the same day the blood was drawn. Blood samples were serially coded, and 2–4 samples were blind tested every week. The whole study took almost 2 yr.

Colony formation

The cells were incubated at 37°C in 95% air/5% CO_2 at 100% humidity. After 1 wk, 0.1 mL of the medium was removed from each well and fresh medium containing 0.2 ng/mL of recombinant IL-2 was added. Two weeks after the irradiation, the presence or absence of lymphocyte colonies was determined for each well using an inverted microscope. Wells were scored positive if they contained growing lymphocytes on the periphery of the feeder cell mass at the center of the round well.

Dose-survival study of log-phase irradiation

All the colonies appearing in the nonirradiated control plate were collected, mass cultured for several days, and then stored in liquid nitrogen. A total of 14 individuals whose G_0-irradiated lymphocytes showed apparent radioresistant or radiosensitive survival curves were selected. The stored lymphocytes from these individuals were thawed and cultured for several days in the presence of feeder cells. These lymphocytes were used for the log-phase-irradiated dose-survival tests essentially as described above, except that the average number of cells

seeded in each well was increased, namely, 2 cells for controls, 4 cells for 1 Gy, 6 cells for 2 Gy, 30 cells for 3 Gy, 100 cells for 4 Gy, and 300 cells for 5 Gy. The results were analyzed using a linear equation instead of the linear-quadratic equation used for fitting the data of G_0-irradiated lymphocytes (see below). In each experiment, lymphocytes from one apparent radioresistant and one apparent radiosensitive case were selected and tested. Lymphocytes from each donor were tested twice with a different combination of donors.

Micronucleus induction study

A portion of the lymphocytes prepared for the G_0-irradiated dose-survival study was used for the micronucleus induction tests. For each donor, approximately 5×10^4 cells were distributed in each well of a 96-well microtiter plate (flat bottom, 4 wells per donor) with 0.1 mL of medium and were exposed to 3 Gy of X rays. Subsequently, 0.1 mL of medium containing PHA was added. The control plate consisted of the same number of cells without X-irradiation. The cells were incubated for 3 days and were treated with cytochalasin-B (3 μg/mL) during the last 24 hr to block cytokinesis[16]; slide preparations were made by cytospin. Cells were stained with May-Grunwald-Giemsa, and the frequency of micronuclei was determined as described previously.[17]

Statistical considerations

For the G_0- and log-phase lymphocyte studies, if one assumes that the number of cells seeded into an individual well follows a Poisson distribution with dose-dependent mean stated above and that individual cells in a well are independent of each other with respect to survival, the maximum likelihood estimate of the cloning efficiency (CE, the probability of cell survival) obtained from a multiple well experiment is

$$CE = \frac{-\ln(\text{fraction of wells without a colony})}{\text{average number of lymphocytes seeded per well}} .$$

If one assumes that causes of cell death related to culture conditions are independent of causes of cell death related to radiation exposure, the cloning efficiency of cells exposed to dose D, CE_D, is the product of the zero-dose cloning efficiency and the surviving fraction of cells irradiated with dose D; ie,

$$CE_D = CE_0 \, SF_D,$$

where $SF_0 = 1$. The SF_D and CE_0 were modelled using the exponential functions

$$SF_D = \exp(-aD^2 - bD)$$

$$CE_0 = \exp(-c) .$$

Estimates of the coefficients a, b, and c were obtained by fitting the model

$$-\ln CE_D = aD^2 + bD + c$$

using weighted least squares, with weight proportional to

$$[\ln(\text{average number of cells per well})]^{-1/2}$$

to compensate for the larger variance in $\ln CE_D$ at higher doses.

On the basis of this equation, the D_{10} value (the X-ray dose required to kill 90% of the cells) was calculated. Coefficient of variation (CV; mean/standard deviation) was used to express the distribution of D_{10} or other biological parameters.

X-irradiation

The X-ray machine (Shimadzu Co, Model WSI-250S, Kyoto) was operated at 220 kVp, 8 mA with an Al filter 0.5 mm thick and a Cu filter 0.3 mm thick. The dose rate was about 0.25 Gy/min. The total dose was measured for all the experiments using a Victoreen 500 dosimeter (Victoreen Inc, Cleveland, Ohio) calibrated at the Research Institute for Nuclear Medicine and Biology of Hiroshima University. Cells were irradiated at room temperature.

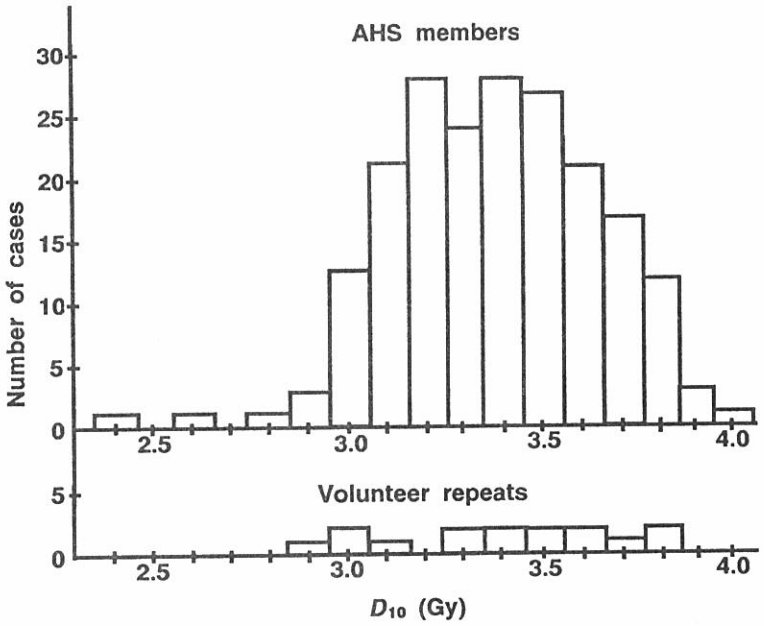

Figure 1. D_{10} values for the single tests of G_0-irradiated lymphocytes from 201 members of the Adult Health Study and from one reference donor tested 15 times.

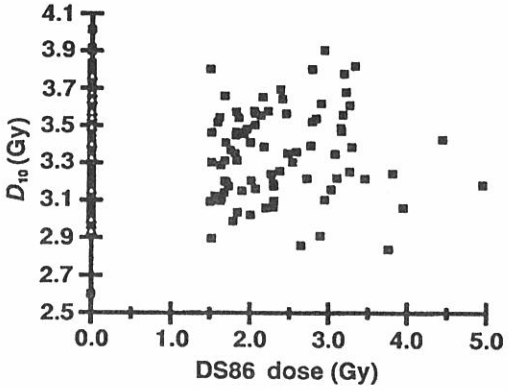

Figure 2. D_{10} values for single tests of G_0-irradiated lymphocytes from 201 members of the Adult Health Study, according to their Dosimetry System 1986 doses.

Results

Lymphocytes from 201 AHS participants were tested during a period of about 2 yr. Fifteen repeat assays were also performed on one donor aged 42 yr over the same period. During a period of about 6 wk, 12 consecutive cases showed unusually low CE_0 values due to unidentified problems with the medium. However, since CE_0 was essentially independent of D_{10} values and other measures of sensitivity, no similar pattern was seen in D_{10}, and these experiments were retained in the analysis. Excluding them had no effect on the results. The estimated CE_0 ranged from 0.092 to 0.90 (median = 0.45) in the 201 AHS participants and from 0.10 to 0.61 (median = 0.49) in the 15 repeat tests.

The mean estimated D_{10} (CV) was 3.37 Gy (7.7%) in the 201 AHS members and 3.39 Gy (8.2%) in the 15 repeat tests, with ranges from 2.40 to 4.01 and 2.93 to 3.76, respectively. Figure 1 shows the distribution of D_{10} values for the two groups.

Figure 2 shows the D_{10} values plotted as a function of total Dosimetry System 1986 (DS86) kerma dose. There is no apparent trend. Linear regression analysis of D_{10} on kerma dose for the 201 AHS members gives a slightly negative but nonsignificant slope (-0.017 ± 0.014, $p > .2$). There was no significant effect of either age or sex on D_{10} values (data not shown). In other words, there were no signs of preferential elimination of individuals with higher cellular radiosensitivity from the high-dose survivors. In addition, severe epilators, who reported loss of more than two-thirds of their hair, in the high-dose group ($n = 47$) were compared with nonsevere epilators and nonepilators combined in the high-dose and the control groups ($n = 149$) since such a biological difference may be caused by a difference of cellular radiosensitivity of the individuals. The mean D_{10} value in the epilators was 3.32 Gy, which was not significantly different from 3.39 Gy in the others; ie, there was no evidence that lymphocytes from severe epilators were more radiosensitive.

Note also that, since the CV for 201 AHS members was essentially the same as that for 15 repeat tests on one volunteer, the observed variation among individuals in measures of cellular radiosensitivity appears to be mostly attributable to experimental errors. Intrinsic heterogeneity in radiosensitivity is comparatively quite small, if it exists at all. To confirm this conclusion, two additional sets of experiments were undertaken.

The first examined dose-survival responses of lymphocytes irradiated under growing conditions instead of being irradiated at G_0. The rationale is that if there were individual differences in the repair speed of DNA damage, G_0-irradiation

tests may not be sensitive enough to detect them since these cells enjoy a long G_1 period before the onset of DNA replication and, therefore, individual variation might have been diminished.

For this study, 14 individuals were selected randomly, 7 from those with low D_{10} values from the G_0-lymphocyte study and 7 from those with high values. Two experiments were performed for each individual. The results are shown in Figure 3. The mean (CV) D_{10} was 2.88 Gy (12.0%) in the low-D_{10} group and 2.98 Gy (12.4%) in the high-D_{10} group. The difference was not statistically significant. In this experiment there was some evidence of inter-individual variation in D_{10} values after accounting for experimental error ($F_{13,14}$ = 2.43, p = .06). The CV attributable to interindividual variation was 7.9% and that to experimental error 9.3%.

The second experiment concerned the induction of micronuclei. Although there is good evidence for a close association between gross chromosomal damage and reproductive death of G_0-irradiated lymphocytes,[18] the contribution of interphase death is difficult to exclude. If a considerable fraction of G_0-irradiated lymphocytes die via interphase death, the current variation of D_{10} values among individuals would be an underestimate. Therefore, it was thought important to look at DNA damage for those persons whose G_0-irradiated lymphocytes showed apparent radioresistant or radiosensitive dose-survival responses.

Therefore, a portion of the cells used for the G_0-lymphocyte dose-survival experiments was also subjected to 0 or 3 Gy of X-irradiation for the measurement of micronucleus induction. As in the log-phase-irradiation experiments, 18 cases were selected at random, 9 each from high- and low-D_{10} values from the G_0-lymphocyte dose-survival experiments. The results are shown in Figure 4. The mean (CV) induced micronucleus frequency was 0.394 (16%) in the high-D_{10} group and 0.418 (19%) in the low-D_{10} group. This difference also was not significant.

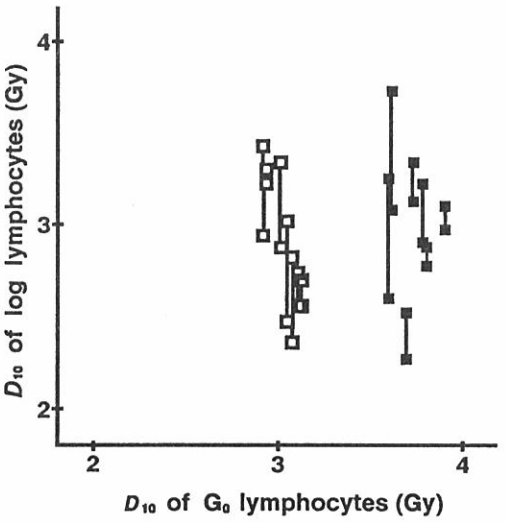

Figure 3. D_{10} values of log-phase-irradiated lymphocytes, according to the D_{10} of G_0-irradiated cells. Closed and open symbols represent apparent radioresistant and radiosensitive cases, respectively, after the G_0-irradiation tests. The vertical bars connecting two points represent results of repeated tests on the same cell line.

Discussion

The present results from the dose-survival tests of G_0-irradiated lymphocytes do not show any difference between the average radiosensitivities of the high-dose and control groups of survivors. Thus, the results provide no evidence for the preferential loss of individuals whose cells were genetically more radiosensitive than those of others among the high-dose group.

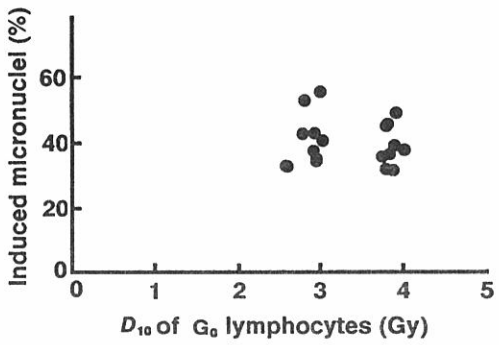

Figure 4. Induced frequency of binucleated cells bearing micronuclei after exposure of G_0 lymphocytes to 3 Gy of X rays in vitro, according to the D_{10} values of G_0-irradiated lymphocytes.

To avoid misunderstanding, it should be mentioned that the major finding of the present study for G_0-irradiated lymphocytes from 201 individuals is that the individual variation supposedly determined by the genetic background is quite small, if it exists at all. This finding had been suggested in our previous study on a smaller sample. Namely, CV values for the mean D_{10} were quite similar among 31 individuals (CV = 5.0%) and 28 repeat tests of a single donor (CV = 5.7%).[19] The values observed in the present study were 7.7% for 201 individuals and 8.2% for 15 repeat tests of the same donor. The slightly smaller CV value for the previous 31 individuals compared with that for the present 201 individuals may simply be due to the random drift associated with the smaller sample. Alternatively, because the present study took considerably longer—over 2 yr, it is also conceivable that the experiments may have been subjected to some confounding factor(s) to a greater extent. In any event, the CV values for the 201 individuals were not distinctively larger than those attributable to experimental errors estimated from concurrent control groups consisting of repeat tests of a single donor. It is also mentioned that the CV value of 7.7% for 201 individuals is not at all larger compared with that attributable to interexperimental variation in another type of commonly used cells, human skin fibroblasts (see below).

These considerations lead us to believe that the present conclusion is merely a consequence of the quite small interindividual variation in the present G_0-lymphocyte assay, not an indication that the heterogeneity existed but failed to give rise to a significant difference between the high-dose and control groups.

One may argue that the present lymphocyte assay may not be adequate to detect individual differences in cellular radiosensitivity. However, it is known that the lymphocytes derived from AT patients are highly vulnerable to the killing effects of ionizing radiation, as are the skin fibroblasts.[6,7,20] Similar characteristics are known also for the lymphocytes from xeroderma pigmentosum patients, which are highly sensitive to ultraviolet light.[21] So far, there is no evidence that the lymphocyte colony assay behaves differently from assays of other sources of cultured cells.

The $LD_{50/30}$ of animals has been known to vary not only with strain or species but also with age and other environmental factors. Thus, the present lymphocyte results imply nothing about the presence or absence of heterogeneity in individual survivability among the A-bomb survivors.

To our knowledge, the present study is the first to have tested normal lymphocytes from as many as 200 individuals, and thus it would be worthwhile to review briefly the cellular radiosensitivity studies on apparently normal people. Skin fibroblasts have long been used for this purpose, and Figure 5 summarizes the

results of these studies. Because laboratories use different irradiation facilities and irradiation procedures, results from different laboratories cannot be combined. Therefore, the results from each report are plotted here. The fibroblasts shown here were mainly from apparently normal individuals. However, samples from patients with hereditary diseases but with normal radioresponsiveness are also included, but AT cases are not included. Although different studies use different parameters, such as D_0, D_{10}, or $\overline{D}$, these parameters are not considerd here; the CVs for the means were simply compared.

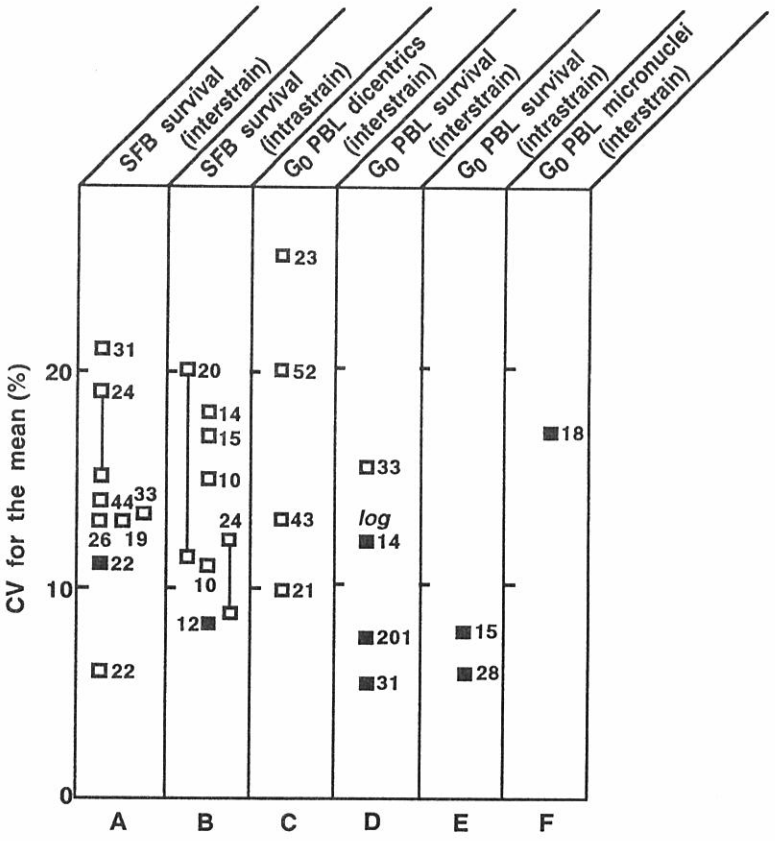

Figure 5. Variation of cellular radioresponses, including dose-survival curves (A, B, D, E), and the induction of dicentric chromosomes (C) and of micronuclei (F). To compare results of different endpoints, the coefficient of variation (CV, ie, the standard deviation divided by the mean) was used to estimate the variation. The closed symbols are our own data, and the open symbols are from published reports. The values next to each symbol represent either the total number of different cell lines tested (A, C, D, F) or the total number of repeat tests for a single cell line (B, E). The vertical bars connecting two points represent results based upon the same data set but using different biological endpoints, ie, D_0 or D_{10}. PBL = peripheral-blood lymphocyte; SFB = skin fibroblast. References: (A) Little et al,[22] Cox and Masson,[23] Arlett and Harcourt,[8] Kushiro et al,[24] Weichselbaum et al,[9] and Little and Nove.[25] (B) Arlett et al,[26] Arlett and Harcourt,[8] Little et al,[22] and N. Nakamura (unpublished observations). (C) Scott,[27] Evans,[28] Liniecki et al,[29] and Sasaki and Tonomura.[30] (D, E, and F) Nakamura et al[19] and present results.

Studies on more than 10 strains of fibroblasts (Figure 5, column A) show that CVs vary from 10% to 20% except for one report. Results from more than 10 repeat tests of the same cell strains (column B) also show CVs of from 10% to 20%. When these two sets of data are compared, the CVs for interstrain tests do not appear to be larger than those for repeated tests of the same strains. That is, as far as the comparison of CVs is concerned, individual cellular radiosensitivity does not appear to vary much. Columns C to F show the results for lymphocytes. Column C is for the induction of dicentric chromosomes, and the CV varies here, also, from 10% to 20%. In the colony assay of peripheral blood lymphocytes (columns D and E), the CVs are smaller than 10% for both interindividual and repeat tests except for the study on log-phase-irradiated cells. Column F shows our results on the induction of micronuclei, CV = 17%. Dicentric and micronucleus induction tests are both based upon single-exposure experiments and not upon fitted curves from multiple-dose points, which would have contributed to the increase of CV values.

Why is there a large difference in the CVs between the fibroblasts and lymphocytes, given that they both use the colony-formation assay? Any answer must explain why the CV is larger in fibroblasts than in lymphocytes even for repeated tests of the same strain of cells. Thus, neither ethnic group differences among the donors, ie, the fibroblast data are for Caucasians and the lymphocyte data are for Japanese, nor possible heterogeneity in the fibroblast population[31] is a satisfactory explanation. Our previous study on the comparison of skin fibroblasts and lymphocytes derived from the same individuals suggested that, although each observed point of the dose-survival experiments with fibroblasts lies closer to the fitted curve compared with those of lymphocytes, the standard deviation for the mean D_{10} value for 22 individuals was about 2 times larger; ie, there was a wider distribution in the fibroblasts than in the lymphocytes.[24]

This implies that the whole fibroblast population is affected systematically by the confounding factor(s) and not randomly in each dose group. The major difference between the fibroblasts and the lymphocytes is that the unstimulated lymphocytes are mostly at the G_0 phase of the cell cycle, whereas the fibroblasts are successively cultured in vitro and are at random growth phases (log phase) at the time of radiation exposure. One report noted changes of radiosensitivity associated with the in-vitro passage of fibroblasts,[32] but the results do not appear to be reproducible,[22] and hence it is difficult to consider this to be the major cause. One of the possible explanations is the different cell-cycle distribution[33] caused by subtle differences in culture conditions and/or unstable expression of repair-related genes in the fibroblasts. In the lymphocytes also, when they were irradiated at log phase, it appears that the CV increases, although the total number of tests is rather limited. It would be interesting to irradiate the fibroblasts under the confluent conditions to see if the interstrain variation diminishes compared with that of cells irradiated at log phase.

The present results contain some implications for the use of peripheral-blood lymphocytes for medical purposes. For example, it has been recognized that patients who undergo a fixed protocol of radiotherapy show various degrees of side effects, and that some of them actually fail to accomplish a whole course due to unusually severe reactions in normal tissues.[34] It has been suspected that their cellular radiosensitivity is abnormally higher than that of others, whereas the

available data from in-vitro fibroblast studies suggest that this is not the sole cause.[35,36] Although the lymphocyte dose-survival assay does not require the precultures that are necessary for the fibroblasts, and hence less time (ie, 10 to 14 d) is needed to collect the information on cellular radiosensitivity, the present results indicate that the G_0-lymphocyte assay under acute irradiation conditions is not capable of providing better information since the individual variation is so small. Because D_{10} values of G_0-irradiated lymphocytes did not show a correlation with the frequency of micronucleus induction, it is also unlikely that the micronucleus tests can provide better information.

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