Measurement of the CD3⁻4⁺ Variant T Cell Frequency by Flow Cytometry after X-Irradiation on Mice

Naoki KUNUGITA, Nan MEI, Satoshi NOMOTO and Toshiyuki NORIMURA

Department of Radiation Biology and Health, School of Medicine, University of Occupational and Environmental Health, Japan

Abstract: Measurement of the CD3⁻4⁺ Variant T Cell Frequency by Flow Cytometry after X-Irradiation on Mice: Naoki KUNUGITA, *et al.* Department of Radiation Biology and Health, School of Medicine, University of Occupational and Environmental Health, Japan—Variant cell frequency of mice T-lymphocytes defective in the T-cell receptor (TCR) gene expression was measured by flow cytometry. Splenic T-lymphocytes were obtained from mice after irradiation and stained with fluorescein-labeled anti-L3T4 (CD4) and phycoerythrin-labeled anti-CD3-ε antibodies. They were analyzed with a flow cytometer to detect variant T cells lacking surface expression of TCR/CD3 complexes which showed CD3⁻4⁺ T cells. Variant T cells could be observed 3 days after 3 Gy irradiation. Frequency of variant T cells increased to the maximum level of 94×10^{-4} between 15 and 21 days after 3 Gy irradiation on BALB/c mice and gradually decreased with a half-life of approximately 17 days. We examined the strain difference in radiosensitivity of splenic T-lymphocytes in BALB/c, C57BL/6, C3H/He and B6C3F₁ mouse strains by this assay method. We observed that BALB/c showed the most radiosensitivity. This assay method is quick and easy in comparison with other methods and is considered useful for the mutagenicity test.

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Key words: Strain difference, Radiosensitivity, CD3⁻4⁺ variant T cell, T-cell receptor gene, Flow cytometry

It is well known that somatic mutations are induced by ionizing irradiation. Many investigators have reported various different mutagenicity tests which include gene mutation, chromosomal aberration and DNA damage¹, but it is difficult to detect

somatic mutations quantitatively. It is very important to evaluate the effects of mutagens not only in industry but also in our living environment.

Recently, Kyoizumi *et al.* established the somatic mutation assay at the T-cell receptor (TCR) gene². The TCR is a heterodimer consisting of α and β chains and is associated with a molecular complex referred to as CD3 antigen. Peripheral CD4⁺ lymphocytes deficient in the expression of TCR/CD3 complex on the cell surface show CD3⁻4⁺ due to a somatic mutation at either the TCR α- or β- chain genes. They showed that the mean frequency of T cells with altered TCR expression was 2.5×10^{-4} among CD4⁺ mature T cells in peripheral blood from healthy donors². They also showed that the frequency of T cells with altered TCR expression significantly increased with age among normal donors², increased in Thorotrast patients who had been continuously irradiated with α particles³ and increased in a dose-dependent manner among radiation-exposed donors treated with ¹³¹I for thyroid disease⁴. They showed that this assay may be useful for estimating the effects of mutagens as a biological dosimeter and for identifying high-risk cancer groups⁵.

On the other hand, mouse strain differences in radiosensitivity have been widely observed and are considered to be determined by several factors⁶⁻¹³. Till and McCulloch demonstrated the radiation sensitivity of mouse bone marrow cells in C57BL/Ha and C3Hf/Ha strains⁶. In respect to the median lethal dose for 30-day death (LD_{50/30}), BALB/c was the most radiosensitive⁷⁻¹⁰. Radiosensitivity of lymphocytes has also been observed in a variety of end points and shown to differ in diverse strains of mice^{11, 12}.

The present investigation was undertaken to detect variant T cells lacking surface expression of TCR/CD3 complexes in mouse splenocytes after irradiation and to examine the strain difference in

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Correspondence to: N. Kunugita, Department of Radiation Biology and Health, School of Medicine, University of Occupational and Environmental Health, Japan, 1-1 Iseigaoka, Yahatanishi-ku, Kitakyushu 807, Japan

radiation-induced $CD3^{-}4^{+}$ variant T cell frequency. We found that BALB/c mice are extremely radiosensitive in this assay. Furthermore, this TCR mutation assay method using flow cytometry is very easy and useful for screening the effects of *in vivo* exposure to toxic and/or mutagenic agents as well as radiation.

Materials and Methods

Animals: Seven-week-old female BALB/c, C57BL/6 and C3H/He mice and F_1 hybrids B6C3F₁ were purchased from Japan SLC, Inc. (Shizuoka, Japan). After arrival, the mice were kept for a week to check their body weight and for infections, and then used for the experiments. They were housed, 10 mice per cage, on wood-chip contact bedding in a $23 \pm 2^{\circ}\text{C}$ air-conditioned clean room and given commercial pellet chow and water *ad libitum*. Research was conducted according to the principles enunciated in the "Guiding Principles for the Care and Use of Animals approved by the Faculty Meeting of UOEH".

Irradiation: Mice were placed in a special plastic box and exposed to X-rays using a Toshiba EXS-300-4 X-ray apparatus (Toshiba Co., Ltd., Tokyo, Japan) in an unanesthetized condition. The X-irradiation factors were 250 kVp, 0.5 mm Cu + 0.5 mm Al filter, 50 cm focus-surface distance and the dose rate was 0.45 Gy/min.

Flow cytometry: Mice were sacrificed 3 to 50 days after irradiation and the spleen was sampled immediately after sacrifice. The spleen cells were gently dissociated and then filtered through a stainless steel

mesh. Red blood cells in the spleen cells were lysed with a Gey's solution in which NaCl was replaced by NH_4Cl . T cells were enriched through nylon wool columns according to Julius *et al.*¹⁴⁾. These cells, which were nylon wool non-adherent cells, contained 75 to 90% T cells.

One million cells were stained on ice for 30 min. with fluorescein-labeled anti-L3T4 (CD4) and phycoerythrin-labeled anti-CD3- ϵ antibodies which were obtained from PharMingen (San Diego, CA, U. S. A.). Then they were washed and analyzed with a Coulter EPICS XL flow cytometer (Coulter Corporation, Hialeah, FL, U. S. A.). Lymphocytes were examined by setting bit map gates on a plot of forward and 90° light scatter, and a window for $CD3^{-}4^{+}$ variant T cells was set in the region where the surface CD3 level was $< 1/25$ th of that for normal $CD4^{+}$ cells according to Kyoizumi *et al.*²⁾ (Fig. 1). The variant T cell frequencies were calculated as the number of events in the $CD3^{-}4^{+}$ T cell window divided by the total number of $CD4^{+}$ T cells.

Statistical analysis: The statistical significance in the difference between variant T cell frequencies among strains was determined by one-way analysis of variance and the statistical significance between two strains was determined by the least significant difference test.

Results

Fluorescence distributions of TCR variants after X-irradiation: Fig. 1 shows the results of flow-cytometric distributions of $CD3^{-}4^{+}$ variant T cells

Fig. 1. Flow cytogram of splenic T lymphocytes from a normal BALB/c mouse and the mouse 17 days after 3 Gy irradiation stained with fluorescein-labeled anti-L3T4 (CD4) and phycoerythrin-labeled anti-CD3- ϵ antibodies. A window for variant $CD3^{-}4^{+}$ lymphocytes is shown as the square.

from normal BALB/c mice and the mice 17 days after 3 Gy irradiation. A window for CD3⁻CD4⁺ variant T cells was set in the region shown as squares in the figure.

Determination of the length of the expression time for TCR variants: Before assaying the mutagenic effects of X-irradiation as a function of the dose, we determined the optimal length of time for the expression of TCR variants. T cells with altered TCR expression could be observed 3 days after 3 Gy irradiation. Variant T cell frequency increased to the maximum levels between 15 and 21 days after 3 Gy irradiation and gradually declined with a half-life of almost 17 days (Fig. 2).

Strain differences in the radiosensitivity at the TCR gene: Frequencies of CD3⁻CD4⁺ variant T cells after irradiation in each strain are shown in Fig. 3. Each mouse was sacrificed 15 to 17 days after irradiation. In all strains, CD3⁻CD4⁺ variant T cells were increased in a dose-dependent manner. Variant T cell frequency after irradiation in BALB/c

Fig. 2. Time course of variant T cell frequency after 3 Gy irradiation in BALB/c mice.

Fig. 3. Dose response curve of variant T cell frequency 15 to 17 days after irradiation in BALB/c, C3H/He, B6C3F₁ and C57BL/6 mice.

mice was significantly higher than in the other strains. Strain difference in induced CD3⁻CD4⁺ variant T cell frequency after 3 Gy is shown in Fig. 4. Induced variant T cell frequencies after 3 Gy irradiation on BALB/c, C3H/He, B6C3F₁ and C57BL/6 mice were 59.7, 29.8, 21.8, 16.6 ($\times 10^{-4}$), respectively. BALB/c mice showed the most radiosensitivity among these four strains and were induced at almost twice the variant T cell frequency of C3H/He mice. C57BL/6 mice were the most resistant to 3 Gy irradiation.

Discussion

In this study, we have measured flow-cytometrically the variant cell frequencies of mouse T-lymphocytes which were defective in the TCR gene expression after whole body irradiation. Splenic T-lymphocytes were obtained from the mice after irradiation and stained with fluorescein-labeled anti-L3T4 (CD4) and phycoerythrin-labeled anti-CD3- ϵ antibodies. Variant T cells lacking surface expression of TCR/CD3 complexes showed CD3⁻CD4⁺ T cells in a flow cytometer. They could be observed 3 days after 3 Gy irradiation. The frequency of variant T cells increased to the maximum level of 94×10^{-4} between 15 to 21 days after 3 Gy irradiation on BALB/c mice and gradually decreased with a half-life of approximately 17 days. Induced variant T cell frequencies after irradiation on BALB/c and C3H/He mice were about 20×10^{-4} , 10×10^{-4} per Gy, respectively. In human cases who were patients recently treated with ¹³¹I for thyroid cancer, which was equivalent to a committed ¹³¹I dose of 1.05 Sv to the spleen, the net increased mutation frequency was 10×10^{-4} . The data in this study

Fig. 4. Comparison of the induced frequency of variant T cells by 3 Gy of X-irradiation in BALB/c, C3H/He, B6C3F₁ and C57BL/6 mice.

*; $p < 0.05$, **; $p < 0.01$.

of mouse lymphocytes may therefore agree with the data for human lymphocytes.

Kyoizumi *et al.* assumed that variant CD3⁺ CD4⁺ T cells are generated by somatic mutation of productively rearranged TCR genes in peripheral mature T cells²⁾. Therefore the half-life of 17 days was probably reflected in the life span of mature T-lymphocytes *in vivo* in the mice. Another possibility is the elimination of these variant T-lymphocytes *in vivo*, because Kyoizumi *et al.* also showed that these mutant T-lymphocytes generally grow poorly *in vitro*²⁾.

Mouse strain differences in radiosensitivity were widely observed and are considered to be determined by several factors⁶⁻¹³⁾. BALB/c mice showed the most radiosensitivity and were induced at almost twice the variant T cell frequency of C3H/He mice in this study. It has been reported that BALB/c is one of the most radiosensitive strains shown by any assay method^{7, 10, 12, 13, 15, 16, 18)}. With respect to the median lethal dose for 30-day death (LD_{50/30}), BALB/c was the most radiosensitive⁷⁻¹⁰⁾. The LD_{50/30} values for BALB/c, C3H/He and C57BL/6 were 500.1R, 593.2R and 630.3R, respectively⁸⁾. Other reports showed the same tendency in LD_{50/30} values^{7, 9, 10)}. Radiosensitivity of lymphocytes has also been observed by a variety of end points and has been shown to differ among strains of mice^{11, 12, 15)}. Recently, Mori *et al.* showed that thymocyte from BALB/c mice is more sensitive to radiation-induced apoptosis¹⁵⁾. They also reported that thymocytes from C57BL/6 mice showed a similar radiosensitivity to BALB/c thymocytes, while C3H/He mice exhibited a resistant phenotype. We have also shown the strain difference in radiosensitivity in mice observed by colony forming assay with T-cell growth factor^{17, 18)}. The splenic T-lymphocyte of BALB/c was most sensitive to radiation among BALB/c, C3H/He, C57BL/6 and B6C3F₁ mice¹⁸⁾. For BALB/c, C3H/He, C57BL/6 and B6C3F₁ mice, respectively, the D₀ values were 0.64 Gy, 0.97 Gy, 0.75 Gy and 0.79 Gy, while the D_q values were 0.63 Gy, 0.62 Gy, 0.51 Gy and 0.71 Gy. It has also been reported that C57BL/6 is almost as radiosensitive a strain as BALB/c mice^{12, 15)}. Sado *et al.* reported that the D₀ and D_q values in the radiosensitivity of plaque-forming cells (PFC) per spleen were 1.45 Gy and 0.62 Gy for C3H, 0.42 Gy and 0.53 Gy for BALB/c and 0.15 Gy and 0.58 Gy for C57BL/6 mouse, respectively¹²⁾. In this study, however, C57BL/6 was most resistant to irradiation. The reason for this discrepancy was not found and further experiments are needed to solve this problem.

On the other hand, we have observed the frequency of TCR mutants and 6-thioguanine-resistant cells determined by a cloning at the hypoxanthine phosphoribosyltransferase (HPRT) gene after treatment with 250 kVp X-rays or ethylnitrosourea (ENU) on human lymphocytes *in vitro*. There was a linear relationship between mutation induction on TCR genes and the HPRT gene after both treatments¹⁹⁾.

In conclusion, this assay is a very useful method to measure the variant cell frequencies not only for human lymphocytes but also for mouse lymphocytes, because it requires a small number of cells, a short period for the measurement and antibodies are commercially available.

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