Analysis of Micronuclei in the Transferrin-receptor Positive Reticulocytes from Peripheral Blood of Nasopharyngeal Cancer Patients Undergoing Radiotherapy by a Single-laser Flow Cytometer

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Micronuclei/Immunomagnetic isolation/Acridine orange/Flow cytometry/Radiation.

The automated micronucleus test is now accepted as a simple, objective, and accurate method for evaluating potential mutagenic effects caused by physical, chemical or biotic factors. This paper describes a single-laser flow cytometry, based on an immunomagnetic isolation technique in combination with acridine orange staining, to detect frequencies of micronucleated transferrin-receptor positive reticulocytes from human peripheral blood. Using this flow cytometric system, we detected the frequencies of micronucleated transferrin-receptor positive reticulocytes from 10 nasopharyngeal cancer patients undergoing radiotherapy and the baseline of the frequencies of micronucleated transferrin-receptor positive reticulocytes from 7 healthy donors. The results showed that the mean frequency of micronucleated transferrin-receptor positive reticulocytes from healthy donors was 0.236% and that from nasopharyngeal cancer patients before radiotherapy was 0.297%. After radiotherapy it was significantly elevated. When the cumulative dose of radiotherapy was about 20Gy, it reached a maximum of 6.905%, and then, as the cumulative dose of radiotherapy continued to increase to 30Gy, 40Gy and 50Gy, the frequency decreased to 6.258%, 5.119% and 5.007% respectively. Our results indicated that the single-laser flow cytometric system was quick, reasonable and acceptable for detecting the frequency of micronucleated transferrin-receptor positive reticulocytes from human peripheral blood.

INTRODUCTION

It is widely accepted to screen chemicals for clastogenic and/or aneuploidogenic effects with the rodent in vivo micronucleus test.¹,²) The earliest in vivo micronucleus test used bone marrow from mice or rats.³–⁵) Since it was first reported that peripheral micronucleated erythrocytes would not be destroyed by mice spleen,⁶) many investigators have employed peripheral blood from mice for micronucleus test. Employing peripheral blood has at least two advantages over using bone marrow. Firstly, it enables the investigators to sample repeatedly from the same animal since the assay does not require the animals to be sacrificed. Secondly, sampling preparation becomes easier and less time-consuming.

However, for a long time, human cytogenetic biomonitoring had been limited within the cytokinesis-block micronucleus test,⁷,⁸) due to the elimination of micronucleated erythrocytes by human spleen. Therefore, splenectomized donors were often used for estimating the contribution of nutritional factors, living styles, radiotherapy or chemotherapy to cytogenetic damage.⁹–¹⁷) So, there were two main obstacles to use the reticulocyte population in peripheral blood from healthy donors for micronucleus test. One was from the human spleen’s elimination of micronucleated erythrocytes, and the other was from the rarity of reticulocytes and extremely low frequencies of micronucleated reticulocytes in human peripheral blood. Theoretically, it is possible to employ reticulocytes in human peripheral blood as the target cell population for micronucleus test if all erythrocytes including micronucleated erythrocytes can be screened, absorbed, counted before the micronucleated erythrocytes are destroyed by the human spleen and if a high-throughput scoring system for micronucleus test can be set up. One of the most important breakthroughs was made by Grawe et al.¹⁸) and further improved by Cao et al.¹⁹) who developed an high-speed automatic flow cytometric method using thiazole...
orange to discriminate reticulocytes from normochromic erythrocytes while Hoechst 33342 was used to discriminate micronucleated erythrocytes from non-micronucleated erythrocytes of mice. This method could, however, not be applied to human peripheral blood because of the rarity of reticulocytes and, especially, the extremely low frequency of micronucleated erythrocytes. In order to overcome this problem, an immunomagnetic technique for isolating and enriching reticulocytes was introduced into micronucleus test to analyze micronuclei in transferrin-receptor positive reticulocytes from human peripheral blood using a dual-laser flow cytometer. However, the dual-laser flow cytometer is still a complex instrument, which limits its wide applications in many developing countries. To further simplify the flow cytometric scoring system, many investigators attempted to detect micronuclei with a single-laser flow cytometer instead of a dual-laser instrument. However, the above single-laser flow cytometric scoring systems required large numbers (more than 600,000) of cells to be analysed due to using peripheral blood directly, and moreover, needed malaria-infected erythrocytes as a model to adjust the flow cytometer as well as a long fixation time (more than 24h), making it difficult to process many samples. What is most recommendable here is that several compounds known to induce micronucleus formation with various mechanisms of action were tested by Criswell et al. with a single-laser flow cytometer and one staining dye - acridine orange. But there are no reports about its applications on micronucleus test using human peripheral blood. In this paper, a more practical single-laser flow cytometric method employing an immunomagnetic isolation technique in combination with acridine orange staining was presented for detecting micronucleated transferrin-receptor positive reticulocytes in peripheral blood from nasopharyngeal cancer patients undergoing radiotherapy as well as from healthy donors.

**MATERIALS AND METHODS**

**Reagents**

Anti-CD71 magnetic bead kit was purchased from Miltenyi Biotech GmbH, Germany, while acridine orange from Sigma, St. Louis, MO.

**Human volunteers and blood sampling**

Total seventeen adult subjects were recruited at the second affiliated hospital of The Third Military Medical University, Chongqing, P.R.of China. Ten nasopharyngeal cancer patients and seven healthy non-smoking volunteers were identified. All blood samples were obtained with informed consent when patients received cumulated doses of 4,10, 20,30,40 and 50 Gy. Samples prior to therapy were taken as controls. About 0.5ml peripheral blood sample was obtained at every sampling time (24 hours after accepting the cumulative dose of radiotherapy) by venipuncture into tubes containing EDTA anticoagulant.

**Immunomagnetic isolation of Transferrin-receptor positive reticulocytes**

The procedure for isolation of transferrin-receptor positive reticulocytes mainly follows the description recommended by the magnetic beads manufactuer Miltenyi Biotech with some modification. Briefly, about 40 µl whole blood was added into 2 ml Sorensen’s buffer (0.05M sodium potassium phosphate, pH 6.8, 2 mM EDTA). Suspended cells were set down by centrifuging at 300 × g for 5 min. After washing, the cell pellet was re-suspended in 160 µl Sorensen’s buffer (0.05M sodium potassium phosphate, pH 6.8) containing 0.5% fetal calf serum, immediately followed by adding 20 µl anti-CD71 magnetic beads, and after adequate mixing, cells were incubated at 8°C for 15 min. Cells were again washed by adding 400 µl Sorensen’s buffer to remove the remaining free anti-CD71 magnetic beads and to decrease nonspecific binding. The cell suspension was centrifuged at 300 × g for 10 minutes, and the supernatant was completely removed. The cell pellet was re-suspended in 500 µl Sorensen’s buffer. A positive selection column type MS+ (Miltenyi Biotech GmbH, Germany) was chosen and placed in the magnetic field of a suitable MACS separator (Miltenyi Biotech GmbH, Germany). The column was prepared by washing with 500 µl Sorensen’s buffer. The cell suspension was applied onto the column. The transferrin-receptor negative cells passed through while transferrin-receptor positive cells were retained. The column was rinsed with 500 µl Sorensen’s buffer, which was repeated twice. The column was removed from the separator, and placed on a suitable collection tube. 1 ml Sorensens buffer was pipetted onto the column and firmly flushed out using the plunger supplied with the column. The positive fraction was collected and centrifuged at 300 × g for 10 minutes. The supernatant was carefully aspirated.

**Fixation and staining**

Cell fixation and staining were performed according to Criswell et al. Briefly, 5 ml of Sorensen’s buffer (0.05 M sodium potassium phosphate, pH 6.8) containing 30 mg/ml sodium dodecyl phosphate (SDS, Sigma, St. Louis, MO), and 1% glutaraldehyde (v/v) was added to a 15ml conical tube. While vortexing vigorously, 200 µl of processed cell suspension was added to the fixative solution. Cells were retained in the fixative for 5 min and then centrifuged for additional 5 min at 300 × g. The supernatant was aspirated, and the pellet re-suspended in 0.2 ml Sorensens buffer.

The staining procedure involved two key solutions - Solution A and Solution B. Solution A was prepared by adding 0.1 ml Triton X-100 (Sigma, St. Louis, MO), 8 ml 1.0 N HCl, and 0.877 g NaCl and distilled water to a final volume of 100 ml. Solution B contained 37 ml 0.1 M anhydrous citric acid, 63 ml 0.2 M Na2HPO4 (pH 6.0), 0.877 g NaCl, 34
mg EDTA disodium salt and 0.6 ml acridine orange from a stock solution of 1 mg/ml. Solution A and B were chilled on ice prior to use. 400 µl Solution A and 1.2 ml Solution B were added into the fixed cell suspension. Tubes were mixed by gentle inversion and allowed to stain on ice for 30 min in the dark. Then, tubes were centrifuged at 300 × g for 5 min. The supernatant was carefully aspirated so that the cellular pellet was undisturbed. The cellular pellet was re-suspended in 0.5 ml Sorensens buffer, ready for flow cytometric analysis.

**Flow cytometric analysis**

Samples were analyzed on a Coulter XL single-laser flow cytometer (Coulter, Hialeah, FL) equipped with a 15 mW argon laser. The forward angle scatter (FS, log scale, related to cell size) and RNA fluorescence (FL4, log, red fluorescence, 675 nm) were chosen as proposed by Criswell et al.\(^{25,26}\) An analysis rate of approximately 1000 cells per second was used. Enumeration of micronucleated transferrin-receptor positive reticulocytes and micronucleated normochromatic erythrocytes was performed using a contour plot (FS vs FL4). To obtain the percentage of micronucleated transferrin-receptor positive reticulocytes within the transferrin-receptor positive reticulocyte region, a gate was set around the transferrin-receptor positive reticulocytes (including normal transferrin-receptor positive reticulocytes and micronucleated transferrin-receptor positive reticulocytes) to exclude the normochromatic erythrocytes (including micronucleated normochromatic erythrocytes) and nucleated cell populations (gate R1, Fig. 3), and then, amorphous gating of the visually apparent micronucleated transferrin-receptor positive reticulocyte region (gate R2) was used. The process was repeated with appropriate gating on the normochromatic erythrocytes (gate R3) and micronucleated normochromatic erythrocytes (gate R4) populations to determine the micronucleated normochromatic erythrocyte percentage.

![Flow Cytometric Analysis](image)

**Fig. 1.** Typical flow cytometric histograms of human peripheral blood samples with (B,C,D) or without (A) immunomagnetic isolation. NCE, Trf-Ret and NC represent normochromatic erythrocytes, transferrin-positive reticulocytes and nucleated cells, respectively. A, B, C and D were from the same cancer patient. A and B were from the same blood sample before radiotherapy. A) whole blood. B) sample isolated. C) sample isolated when cumulative dose reached 20Gy. D) sample isolated when cumulative dose reached 40Gy.
Manual Micronucleus enumeration

For manual micronucleus enumeration, blood samples processed by immunomagnetic technique were collected and acridine orange supravital staining was done upon them. Briefly, blood samples processed by immunomagnetic technique were collected, centrifuged at 300 × g for 5 min. Most supernatant was carefully aspirated and about 10 µl was left following an adequate mixing. Then about 5 µl mixture was applied on an acridine-orange-coated slide and the frequency of micronucleated transferrin-receptor positive reticulocytes was calculated by counting 1000 transferrin-receptor positive reticulocytes through a fluorescent microscope (BX-60, Olympus).

Fig. 2. Variation of the ratios of transferrin-receptor positive reticulocytes (Trf-Ret) to total cells isolated from four healthy donors sampled repeatedly at different times over 40 days.

Fig. 3. Flow cytometric contours showing gating used to analyze micronucleated transferrin-receptor positive reticulocytes (MN-Trf-Ret) and micronucleated normochromatic erythrocytes (MNNCE) populations. R1: all transferrin-receptor positive reticulocytes (Trf-Ret), R2: micronucleated transferrin-receptor positive reticulocytes (MN-Trf-Ret), R3: all normochromatic erythrocytes (NCE), R4: micronucleated normochromatic erythrocytes (MNNCE). A) 0Gy B) 10Gy C) 20Gy D) 40Gy
RESULTS

Isolation and enrichment of transferrin-receptor positive reticulocytes from peripheral blood

Typically, about 50% of the total isolated cells are transferrin-receptor positive reticulocytes which amount up to 200,000 cells for healthy donors and cancer patients in the earlier radiotherapy phase, with the ratio of transferrin-receptor positive reticulocytes ranging from 20% to 90%. We suppose 1 µl of blood contains 5,000,000 erythrocytes and 1% of the erythrocytes are reticulocytes of various types. That means, 10% of total reticulocytes were isolated and 99.9% of total normochromatic erythrocytes removed. The flow cytometric analysis of cell samples before and after immunomagnetic isolation is presented in Fig. 1. The reticulocytes enriched by magnetic separation are the youngest cells (transferrin-receptor positive reticulocytes) among the reticulocyte population. The remaining normochromatic erythrocytes are due to nonspecific binding of the antibody and insufficient washing. The ratios of transferrin-receptor positive reticulocytes to the total cells isolated vary between different donors. Generally, samples from aged donors yielded lower ratios, while young female donors produced

Fig. 4. Flow cytometric dot plots showing gating used to analyze micronucleated transferrin-receptor positive reticulocytes (MN-Trf-Ret) using FL1 (DNA fluorescence, green, 525nm) and FL4 (RNA fluorescence, 675nm). NC, MN-Trf-Ret and Trf-Ret represent nucleated cells, micronucleated transferrin-receptor positive reticulocytes(R3) and total transferrin-receptor positive reticulocytes(R2); both A and B were from the same blood sample of a nasopharyngeal cancer patient after accepting radiotherapy of 10Gy cumulative dose, only with the difference that A was a whole blood sample while B was an immunomagnetically isolated sample.

Fig. 5. The dose-response relationship of the frequencies of micronucleated transferrin-receptor positive reticulocytes (MN-Trf-Ret) of several cancer patients undergoing radiotherapy.

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the highest ratios (data not shown). No differences were observed in samples obtained at different sampling times from the same healthy donor (Fig. 2). The ratio of isolated transferrin-receptor positive reticulocytes as well as that of nucleated cells to the remaining normochromic erythrocytes decreased sharply as radiotherapy advanced into late phase (Fig. 3), which is in agreement with other reports, implying stem cell toxicity, which is also suggested by the decrease of RNA content of transferrin-receptor positive reticulocytes (Fig. 3).

**Flow cytometric and manual determination of micronucleated transferrin-receptor positive reticulocyte frequencies**

In our earlier study, we attempted to distinguish different cell populations through FL1 (DNA fluorescence, green,

| Donor | male/female | age | frequency of MN-Trf-Ret (%) at different cumulative dose (Gy) |
|-------|-------------|-----|------------------------------------------------------------|
| 1     | F           | 29  | 0.35 2.79 3.88 7.53 3.08 4.14 3.89                      |
| 2     | F           | 37  | 0.34 2.97 4.92 7.18 8.08 4.89 5.14                      |
| 3     | F           | 43  | 0.27 2.56 5.94 7.56 6.55 3.51 3.23                      |
| 4     | F           | 41  | 0.23 3.57 6.57 7.64 7.43 6.26 4.97                      |
| 5     | F           | 57  | 0.41 3.29 5.47 6.76 7.21 5.33 4.97                      |
| 6     | M           | 36  | 0.29 2.25 4.62 5.96 5.14 5.61 4.90                      |
| 7     | M           | 38  | 0.36 2.54 5.09 6.11 6.93 5.82 6.00                      |
| 8     | M           | 39  | 0.24 3.57 7.96 8.78 7.61 6.52 7.29                      |
| 9     | M           | 46  | 0.17 3.08 7.17 4.61 4.08 3.27 3.65                      |
| 10    | M           | 55  | 0.31 2.69 6.28 6.92 6.47 5.84 6.03                      |

Mean ± SD 0.297±0.072 2.931±0.447* 5.790±1.241* 6.905±1.143* 6.258±1.636* 5.119±1.134* 5.007±1.226*

* Significantly different from control by One-way ANOVA, SPSS11.0 (p < 0.01)

**Table 2.** The frequencies of micronucleated transferrin-receptor positive reticulocytes (MN-Trf-Ret) of nasopharyngeal cancer patients undergoing radiotherapy of different cumulative doses by microscopic inspection.

| Donor | male/female | age | frequency of MN-Trf-Ret (%) at different cumulative dose (Gy) |
|-------|-------------|-----|------------------------------------------------------------|
| 1     | F           | 29  | 0.20 2.10 5.10 7.80 5.80 4.50 3.80                      |
| 2     | F           | 37  | 0.20 2.90 4.70 6.60 7.60 4.70 5.20                      |
| 3     | F           | 41  | 0.20 3.10 6.10 7.90 6.90 6.60 4.90                      |
| 4     | F           | 43  | 0.20 2.90 5.90 7.40 5.50 3.40 3.50                      |
| 5     | F           | 57  | 0.30 2.50 5.00 6.80 6.90 5.10 5.20                      |
| 6     | M           | 36  | 0.10 2.60 4.50 5.40 4.90 5.40 4.60                      |
| 7     | M           | 38  | 0.20 1.50 4.70 6.20 6.40 5.40 5.40                      |
| 8     | M           | 39  | 0.20 3.20 7.60 8.80 7.20 6.10 6.30                      |
| 9     | M           | 46  | 0.00 2.30 6.60 4.70 4.00 3.30 3.20                      |
| 10    | M           | 55  | 0.05 2.90 5.60 6.40 5.60 5.80 6.10                      |

Mean ± SD 0.210±0.129 2.600±0.521* 5.580±0.987* 6.800±1.223* 6.080±1.124* 5.030±1.081* 4.820±1.050*

* Significantly different from control by One-way ANOVA, SPSS11.0 (p < 0.01)

SD = (standard deviation)
525nm) vs. FL4 (RNA fluorescence, red, 675nm). In FL1 vs FL4 histogram, three cell populations are easily discernible (Fig. 4). Nucleated cell population is located on the upper-right position, while transferrin-receptor positive reticulocyte population on the lower-right position, and normochromatic erythrocyte population on the lower-left position. Between nucleated cell population and transferrin-receptor positive reticulocyte population, is theoretically located micronucleated transferrin-receptor positive reticulocyte population.

However, from Fig. 4, it is hard to distinguish micronucleated transferrin-receptor positive reticulocyte population from normal transferrin-receptor positive reticulocyte population accurately, which makes it hard to calculate the frequency of micronucleated transferrin-receptor positive reticulocytes. So, FL4 vs. FS (forward angle scatter, cell size related) was employed to achieve better discrimination (Fig. 3). The gates used to determine the frequencies of micronucleated transferrin-receptor positive reticulocytes and micronucleated normochromatic erythrocytes are shown in Fig. 3. These gates are region 1(R1, including normal transferrin-receptor positive reticulocytes and micronucleated transferrin-receptor positive reticulocytes), region 2(R2, micronucleated transferrin-receptor positive reticulocytes alone), region 3(R3, including normal normochromatic erythrocytes and micronucleated normochromatic erythrocytes), and region 4(R4, micronucleated normochromatic erythrocytes alone). The frequency of micronucleated transferrin-receptor positive reticulocytes and that of micronucleated normochromatic erythrocytes were determined separately by defining R1 and subsequently R3 as 100%, respectively. To obtain consistent gating, samples that showed the largest frequencies of micronucleated transferrin-receptor positive reticulocytes and micronucleated normochromatic erythrocytes were used to draw free-hand gates around R2 and R4. This protocol was then saved so that all samples prepared on the same day could be analyzed using same identical gates. Fig. 3 B, Fig. 3C and Fig. 3D also show contour plots of different cumulative radiotherapy doses.

The results by flow cytometry show that the frequencies of micronucleated transferrin-receptor positive reticulocytes started to increase significantly when sampling at a dose of 4 Gy for all patients after radiotherapy and reached the highest value (mean ± SD: 6.905 ± 1.143) when the cumulative dose of radiotherapy was 20 Gy. As the cumulative dose of

| Table 4. The frequencies of micronucleated normochromatic erythrocytes (MNNCE) of nasopharyngeal cancer patients undergoing radiotherapy of different cumulative doses by flow cytometry |
|---|---|---|---|---|---|---|---|---|
| Donor | male/female | age | frequency of MNNCE (%) at different cumulative dose (Gy) |
| | | | 0 | 4 | 10 | 20 | 30 | 40 | 50 |
| 1 | F | 29 | 0.01 | 0.01 | 0.02 | 0.12 | 0.20 | 0.25 | 0.26 |
| 2 | F | 37 | 0.01 | 0.02 | 0.03 | 0.09 | 0.16 | 0.22 | 0.26 |
| 3 | F | 41 | 0.01 | 0.02 | 0.03 | 0.15 | 0.25 | 0.31 | 0.29 |
| 4 | M | 43 | 0.03 | 0.02 | 0.04 | 0.17 | 0.25 | 0.28 | 0.32 |
| 5 | F | 57 | 0.02 | 0.01 | 0.02 | 0.07 | 0.23 | 0.28 | 0.26 |
| 6 | M | 36 | 0.01 | 0.01 | 0.03 | 0.11 | 0.16 | 0.20 | 0.26 |
| 7 | M | 38 | 0.01 | 0.01 | 0.02 | 0.10 | 0.25 | 0.29 | 0.37 |
| 8 | M | 39 | 0.02 | 0.02 | 0.04 | 0.09 | 0.13 | 0.23 | 0.30 |
| 9 | M | 46 | 0.02 | 0.03 | 0.02 | 0.10 | 0.18 | 0.22 | 0.27 |
| 10 | M | 55 | 0.00 | 0.03 | 0.05 | 0.13 | 0.19 | 0.23 | 0.23 |

Mean ± SD 0.014±0.008 0.018±0.008 0.029±0.011* 0.113±0.030* 0.200±0.043* 0.251±0.037* 0.282±0.040*

* Significantly different from control by One-way ANOVA, SPSS11.0 (p < 0.01 )

SD = (standard deviation)
radiotherapy continued to increase from 30, 40 to 50 Gy, the frequencies of micronucleated transferrin-receptor positive reticulocytes slowly decreased but still showed a high level and remained significantly higher plateau (mean ± SD: 5.007 ± 1.226) than before radiotherapy (mean ± SD: 0.297 ± 0.072). The dose-response relationship of the frequencies of micronucleated transferrin-receptor positive reticulocytes of every cancer patient undergoing radiotherapy is shown in Fig. 5. The mean frequency of micronucleated transferrin-receptor positive reticulocytes from healthy donors seemed to be slightly lower than that from cancer patients before radiotherapy, but analysis by SPSS (Statistical Product and Service Solutions) 11.0 showed no statistically significant difference between them (P = 0.136) as demonstrated in Table 1 and Table 3. For the frequency of micronucleated normochromatic erythrocytes, it was also elevated after radiotherapy. When the cumulative dose of radiotherapy reached 50Gy, the frequency of micronucleated normochromatic erythrocytes was elevated by about 20 times compared to the baseline. Within the range of 0Gy to 50 Gy, the frequency of micronucleated normochromatic erythrocytes continued to increase, different from the frequency of micronucleated transferrin-receptor positive reticulocytes that seemed to decrease slowly after reaching the highest value when the cumulative dose of radiotherapy was 20 Gy. Like the frequency of micronucleated transferrin-receptor positive reticulocytes, there is no significant difference between the frequency of micronucleated normochromatic erythrocytes from healthy volunteers and that from patients before radiotherapy. The data of the frequency of micronucleated normochromatic erythrocytes from patients and healthy donors were shown in Table 4 and Table 5 respectively.

To validate the results by flow cytometry, we performed microscopic inspection of micronucleated transferrin-receptor positive reticulocytes. The data of the frequencies of micronucleated transferrin-receptor positive reticulocytes by microscopic inspection were shown in Table 2. Comparison between any pair (the frequencies of micronucleated transferrin-receptor positive reticulocytes by flow cytometry vs the frequencies of micronucleated transferrin-receptor positive reticulocytes by microscopic inspection) showed no significant difference by Paired-Samples T test.

**DISCUSSION**

Transferrin receptor is located on cell surface, also called CD71 antigen. It binds Fe (Apo)-transferrin. The receptor delivers transferrin with its bound iron to early endosomes by receptor-mediated endocytosis. Transferrin receptor is expressed on activated T and B lymphocytes, macrophages and most dividing cells but not on resting lymphocytes. It is up-regulated during proliferative responses to antigens or mitogens. Transferrin receptor is present on early erythroid cells, yet it is lost as reticulocytes differentiate into normochromatic erythrocytes.28–30) Serke et al. showed that transferrin-receptor positive reticulocytes are the most immature part among the reticulocyte population.31) Dertinger et al. further proved that transferrin-receptor positive reticulocytes are suitable target cells for micronucleus test using human peripheral blood, and that if the sampling time is optimised, the frequency of micronucleated transferrin-receptor positive reticulocytes from human peripheral blood would be a good substitution for bone marrow micronucleus test to estimate cytogenetic damages by acute exposure.21) Abramsson-Zetterberg et al. successfully employed an immunomagnetic isolation technique to enrich transferrin-receptor positive reticulocytes from human peripheral blood and analyzed micronucleated transferrin-receptor positive reticulocytes by a dual-laser flow cytometer.20) In our studies, we adopted the immunomagnetic isolation technique and succeeded in calculating the micronucleus frequencies of the transferrin-receptor positive reticulocytes with a single-laser flow cytometer using acridine orange staining. Our results showed the immunomagnetic isolation technique was simple, reliable and reproducible for enriching transferrin-receptor positive reticulocytes. Variation of yields of the transferrin-receptor positive reticulocytes between donors is due to host factors such as age and sex which were shown to affect the baseline frequencies of micronuclei as well.32,33) From our unpublished data, radiotherapy decreased the yields of transferrin-receptor positive reticulocytes of cancer patients when the doses reached about 30Gy due to stem cell toxicity, which was also suggested by the RNA content of the transferrin-receptor positive reticulocytes and the ratio of nucleated cells to the remaining normochromatic erythrocytes.

In order to determine the frequencies of micronucleated transferrin-receptor positive reticulocytes, we used FS (forward angle scatter, size indicator) combined with FL4(RNA fluorescence) to discriminate micronucleated erythrocytes from non-micronucleated erythrocytes according to Criswell et al.25,26) Having the highest level of RNA, the nucleated

| donor | male/female | age | frequency of MNNCE (%) |
|-------|-------------|-----|------------------------|
| 1     | F           | 35  | 0.01                   |
| 2     | F           | 35  | 0.03                   |
| 3     | F           | 37  | 0.03                   |
| 4     | M           | 29  | 0.02                   |
| 5     | M           | 38  | 0.03                   |
| 6     | M           | 42  | 0.01                   |
| 7     | M           | 47  | 0.01                   |
| Mean ± SD |      |    | 0.02±0.01             |
cell population is located on the highest position in the contour plots, while normochromatc cell population on the lowest position because of its lack of RNA, and the transferrin-receptor positive cell population on the middle position because of its residual RNA (Fig. 3). Due to the dramatic difference on RNA level, the three cell populations (nucleated cell population, normochromatc cell population and transferrin-receptor positive cell population) can be discriminated very effectively. In our study, we did not use FL1 (DNA fluorescence) to discriminate micronucleated erythrocytes from non-micronucleated erythrocytes (normal erythrocytes); instead, we employed FS, which is size-related, to do the job. The hematologic process and/or mechanisms involved in micronucleus formation, and the known actions of radiation, support the use of a size-related discriminator to distinguish micronucleated erythrocytes from non-micronucleated erythrocytes. Under normal conditions, mitotic division of erythroblasts is a synchronized process resulting in a regulated release of uniformly sized erythrocytes. The sizing is so precise that changes in red cell volume are routinely used in the classification of anemias. Micronuclei, or Howell-Jolly bodies as they are referred to hematologically, are often associated with megaloblastic anemia. Furthermore, megaloblastic anemia is an expected toxic effect for radiation. The action of radiation retards DNA synthesis resulting in prolonged cell growth. An excess of cytoplasmic components, particularly hemoglobin, are produced during the delayed time between cell divisions. This results in micronucleated erythrocytes that are larger than normal ones. Most micronucleus flow cytometric procedures utilize long-term fixation which shrinks cells such that small changes in initial volume of the cells could not be detected. The fixation process incorporated in this assay was short-term and produced uniform spherizing of erythrocytes, allowing capitalization on changes in cell volume.

Acridine orange is a metachromatic fluorochrome, and therefore, capable of fluorescent emission at multiple wavelengths. Intercalation of acridine orange into double-stranded nucleic acid produces fluorescence in the green spectrum. In contrast, acridine orange interaction with single-stranded nucleic acids induce a condensation and precipitation product with maximal emission within the red spectrum. Use of chelating agents such as EDTA produces selective denaturation of RNA, ensuring that double-stranded RNA is not misinterpreted as DNA. Darzynskiewicz demonstrated that DNA and RNA could be discriminated by acridine orange in the presence of EDTA in nucleated cells by excitation at 488 nm and emission at 530 nm DNA and 640 nm RNA. They further showed that acidic conditions markedly enhanced the staining of DNA by dissociating nuclear proteins from DNA allowing increased intercalation of acridine orange. The classical automated micronucleus test by flow cytometry employed an RNA-specific dye (thiazole orange) to discriminate reticulocytes from normochromatc erythrocytes while a DNA-specific dye such as Hoechst 33342 to discriminate micronucleated erythrocytes from non-micronucleated erythrocytes. In our study, we once also attempted to employ FL4 (corresponding to thiazole orange) vs FL1 (corresponding to Hoechst 33342) to distinguish different cell populations (Fig. 4). However, in FL4 vs FL1 histogram, micronucleated erythrocytes can not be distinguished from non-micronucleated erythrocytes very effectively. We think there are two possible reasons for that. One is the insufficient denaturation of double-stranded RNA, and the other is the residual mitochondria DNA in reticulocytes. Those two make non-micronucleated reticulocyte population overlap micronucleated reticulocytes. Moreover, the transferrin-receptor positive reticulocytes are the most immature parts of the reticulocyte family, that means they contain more residual mitochondria and double-stranded RNA, which makes the problem more serious.

Prior studies in our laboratory showed that the frequency of micronucleated lymphocytes was significantly higher after radiotherapy and that it was a better indicator for monitoring cytogenetic damage caused by radiotherapy compared with other indexes. In this paper, transferrin-receptor positive reticulocytes were used as target cell population. Radiotherapy was shown to elevate the frequency of micronucleated transferrin-receptor positive reticulocytes considerably and rapidly. The frequency of micronucleated transferrin-receptor positive reticulocytes reached a maximum when the cumulative dose of radiotherapy was about 20Gy. As the cumulative doses of radiotherapy continued to increase, the frequency of micronucleated transferrin-receptor positive reticulocytes decreased but was still found to be significantly higher than before radiotherapy, implying stem cell toxicity and cell proliferation suppression which were also suggested by the RNA content of the transferrin-receptor positive reticulocytes and the ratios of transferrin-receptor positive reticulocytes and nucleated cells to the remaining normochromatc erythrocytes. Interestingly, we found the frequency of micronucleated normochromatc erythrocytes relatively high in cancer patients undergoing late-phased radiotherapy compared to that before radiotherapy (Table 4). Could not the human spleen completely destroy micronucleated erythrocytes? Were the micronucleated normochromatc erythrocytes just released from bone marrow, and did, therefore, not reach the spleen yet? Or did the radiotherapy damage the function of spleen? These questions need to be further investigated.

As pointed out by Criswell et al., the major criticism of this method may be the use of acridine orange. The micronucleated transferrin-receptor positive reticulocytes population, although readily discernible, occurs on the FL4/FS histogram. Recently, manual scoring and flow cytometric determination of micronucleated erythrocytes were compared using vincristine (an aneugen binding to tubulin), chlorambucil (an alkylating nitrogen mustard and, therefore,
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