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CYTOGENETIC DOSIMETRY BY MICRONUCLEUS ASSAY USING PERIPHERAL BLOOD CELLS IS MODIFIED BY THYROID HORMONES

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Cytokinesis-block micronucleus (CBMN) assay is a convenient and easy method of radiation biodosimetry that uses peripheral blood (PB) cells. However, for micronuclei (MN) frequency induced by ionising radiation, a dose–response relationship in abnormal condition, such as in cancer patients, has not been assessed. To clarify the difference between the dose–response curve generated by the CBMN assay in conditions when thyroid hormone levels were normal and during thyroid hormone withdrawal (THW) prior to ^{131}I treatment, 12 thyroid cancer patients who underwent thyroidectomy were studied. The collected PB mononuclear cells were exposed to 0.5–3.0 Gy X-ray irradiation. Under normal conditions, dose dependency and independency of MN frequency were observed in 92 % and 8 %, respectively. In contrast, during THW, the number of patients who showed dose independency significantly increased to 42 % in comparison with control. Furthermore, a higher concentration of serum thyroglobulin in dose-independent patients was observed. These results suggest that MN frequency in cytogenetic dosimetry is affected by thyroid hormones.

INTRODUCTION

Cytogenetic dosimetry is recognised as a valuable dose assessment method that fills the gap in dosimetric technology when individuals not wearing dosimeters have been exposed to ionising radiation[1]. In particular, the cytokinesis-block micronucleus (CBMN) assay is a convenient and easy method that uses peripheral blood (PB) cells. However, for the micronuclei (MN) frequency induced by ionising radiation, a dose–response relationship in abnormal condition of the body, such as in cancer patients, has not been assessed. The authors' previous data have suggested that bioactive factors, such as cytokines and hormones, indirectly influence haematopoietic cell survival and metabolism in response to oxidative stress[2, 3]. Therefore, it is necessary to confirm whether bioactive factors influence CBMN frequency during radiation exposure in the human body.

Differentiated thyroid cancer (DTC) patients have a higher risk for distant metastasis and locoregional recurrence[4]. Thus, they undergo high levels of physiological stress. Thyroglobulin (Tg), thyroid-stimulating hormone (TSH), free triiodothyronine (fT3) and free thyroxine (fT4) in PB serum and hormonal factors are generally monitored to assess the condition of each DTC patient because these factors show dramatic variation during treatment. Investigation of CBMN frequency in these patients will clarify whether thyroid-related hormones can affect CBMN accuracy. In this study, to clarify whether a dose–response relationship exists between MN frequency and thyroid hormone withdrawal (THW), 12 DTC patients receiving thyroid hormone replacement therapy following thyroidectomy were monitored.

MATERIALS AND METHODS

Study population

This study was approved by the Committee of Medical Ethics of Hirosaki University School of Medicine (Hirosaki, Japan) to ensure the welfare and privacy of participants. Twelve DTC patients (mean age, 58.6 y) treated at the Hirosaki University Hospital between December 2012 and April 2014 were enrolled. The clinical characteristics of each patient are presented in Table 1. All patients underwent thyroid and lymph node surgery for local and/or regional lesions. Further, they were subjected to THW and iodine restriction 2 weeks before ^{131}I remnant ablation (Figure 1).

Collection of peripheral blood

After informed consent was obtained, blood sample was collected using a heparin–lithium tube (Beckon Dickinson, Franklin Lakes, NJ) from DTC patients on two occasions: the normal condition of thyroid hormone and THW condition. The buffy coat and serum were prepared from whole blood. Light-density mononuclear PB cells were separated from the buffy

Table 1. Clinical characteristics of the study population.

	Patients
Number of subjects	12
Gender (female/male)	9/3
Age (y)	58.6 (min.: 39, max.: 76)
Tumor node metastasis classification	
T_{1-2}/T_{3-4}	2/10
N_0/N_{1-x}	0/12
M_0/M_1	11/1
Race	Asian (Japanese)

Figure 1. Schematic diagram of PB sampling design of this study. PB was collected from DTC patients 4 weeks and immediately before ^{131}I treatment. ▼: collection point of PB samples.

coat by centrifugation at 200 × *g* for 30 min on a cushion of Lymphosepar I (1.077 g ml^{-1}; IBL, Fujioka, Japan) and were then washed three times with magnesium/calcium-free phosphate-buffered saline.

Enumeration of PB cells

The total number of viable light-density mononuclear PB cells was counted using a fluorescent cell analyser (Aria SORP; Beckon Dickinson). Expression of the CD45 cell surface antigen on light-density mononuclear PB cells was also analysed.

In vitro irradiation

PB mononuclear cells were exposed to X-ray radiation (150 kVp, 20 mA, 0.5-mm aluminium and 0.3-mm copper filters) using an X-ray generator (MBR-1520R-3; Hitachi Medical Co. Ltd., Tokyo, Japan) at a distance of 46.4 cm between the focus and target. These cells were irradiated in cell culture medium. The dose was monitored using a thimble ionisation chamber that was placed next to the sample during irradiation. The dose rate was ~1 Gy min^{-1}.

CBMN assay

The CBMN assay for cytogenetic dosimetry was conducted for further sample preparation and cell enumeration in accordance with the guidelines of the International Atomic Energy Agency[1]. PB mononuclear CD45$^+$ cells (concentration, 1 × 10^6 cells per dish) were placed in 60-mmφ cell culture dishes (Falcon, Corning®, NY) and cultured in Roswell Park Memorial Institute 1640 medium (Life Technologies, Tokyo, Japan), supplemented with 10 % foetal bovine serum (Japan Bio Serum, Fukuyama, Japan) and 2 % phytohemagglutinin (GE Healthcare, Fairfield, CT). The cultures were incubated at 37°C in a humidified atmosphere of 95 % air/5 % CO_2. Cytochalasin B (6 μg ml^{-1}; Sigma Aldrich, Japan) was added to the culture 24 h post- phytohemagglutinin stimulation. After incubation for 70 h, these cells were harvested, fixed with methanol, stained with Hoechst 33 342 (Sigma Aldrich) and mounted on glass slides. Cellular evaluation was conducted using fluorescence/bright-field microscopy at a magnification of 400× (IX71; Olympus, Tokyo, Japan).

Quantitative analysis of thyroid-related hormones

Thyroid-related hormones (TSH, fT3 and fT4) and Tg were analysed using a Cobas 6000 analyser (<e 601>; Roche, Tokyo, Japan).

Statistical analysis

Statistical analysis was performed using Origin scientific graphing and data analysis software (version 9.0; OriginLab Corporation, Northampton, MA, USA) and SPSS statistical software for Windows (version 17.0; IBM, Chicago, IL, USA). Data were compared using the Wilcoxon single-rank test and Student's *t*-test, and a p value of <0.05 was considered statistically significant. The fitting curve of MN frequency was calculated by $Y = c + \alpha D + \beta D^2$. A dose-dependent MN frequency in each sample was defined by standard error of α and β values using chi-squared test and *F*-test[1].

RESULTS

Hormone variation

Thyroid-related hormones in the PB serum of each DTC patient were quantified during hormone replacement therapy (control) and THW conditions (Figure 2). The median concentrations of fT3 and fT4 were significantly higher in the control group than in the THW group (fT3, 3.0 vs. 0.84 pg ml^{-1}; fT4: 1.8 vs. 0.19 pg ml^{-1}, respectively). In contrast, median TSH and Tg concentrations were significantly increased in the THW group compared with those in the control group (TSH: 89.0 vs. 0.42 μU ml^{-1}; Tg: 25.2 vs. 2.6 ng ml^{-1}, respectively). Individual variances were particularly apparent for fT3, TSH and Tg concentrations in the control group and for Tg concentrations in the THW group.

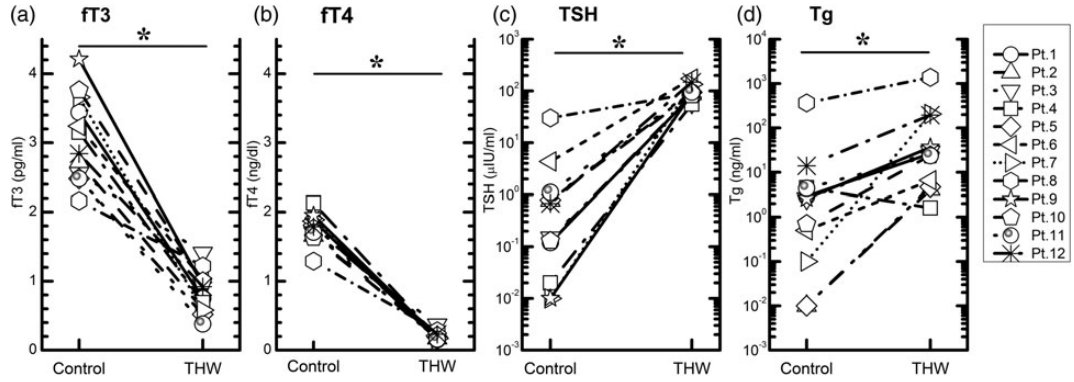

Figure 2. Evaluation of thyroid-related hormones. fT3 (**a**), fT4 (**b**), TSH (**c**) and Tg (**d**) were quantified. $*p < 0.05$ by the Wilcoxon single-rank test.

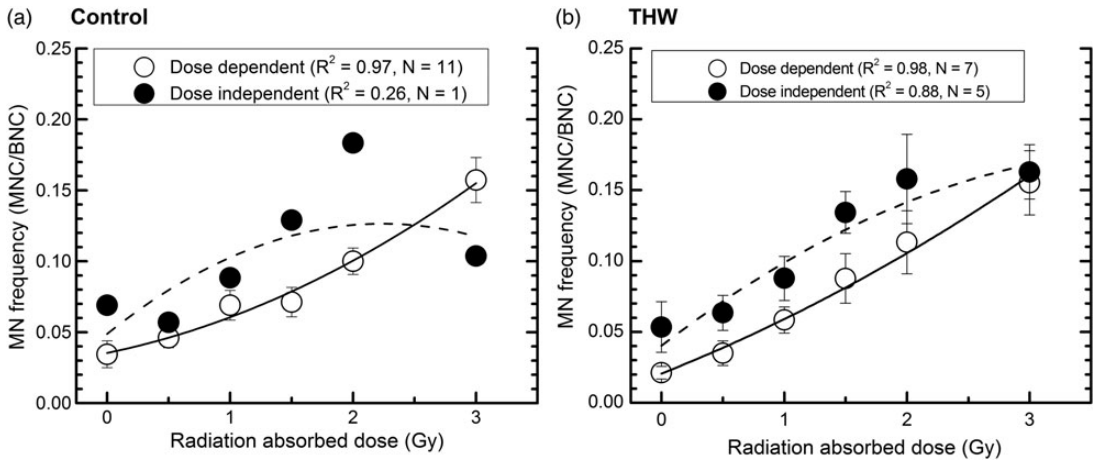

Figure 3. MN frequency in human PB mononuclear cells during control (**a**) and THW (**b**) was compared. The 12 patients were divided into dose-dependent and dose-independent populations.

Radiation dose response of MN frequency

To clarify the difference between the dose–response curves generated by MN frequency, the collected PB mononuclear cells from the control and THW groups were irradiated at 0.5–3.0 Gy (Figure 3). In addition, each patient was classified into having dose-dependent and dose-independent MN frequencies. In the control group, MN frequency was dose dependent in 92 % (11/12), whereas independency was observed in 8 % (1/12) (Figure 3A). In contrast, under THW condition, dose independency of MN frequency significantly increased to 42 % (5/12) in comparison with that of the control (Figure 3B). Furthermore, a similar level of MN frequency was observed in the THW group who received 1.5–3.0 Gy of radiation (Figure 3B). Furthermore, the ratio of Tg variation (THW/control) in the dose-independent group was significantly higher than that in the dose-dependent group (median values of control and THW were 9.1 and 410 ng ml^{-1}, respectively) (Figure 4). Only one patient in the dose-independent group under control condition was also included in the dose-independent group under THW condition.

DISCUSSION

In the present study, to clarify the difference in dose–response curves generated by CBMN assay between a normal condition of thyroid-related hormones and during THW, MN frequency was quantified using PB mononuclear cells in DTC patients. Up-regulation of TSH and Tg followed by down-regulation of fT3 and fT4, which determines the thyroid hormone negative-feedback system[5], was confirmed (Figure 2). This

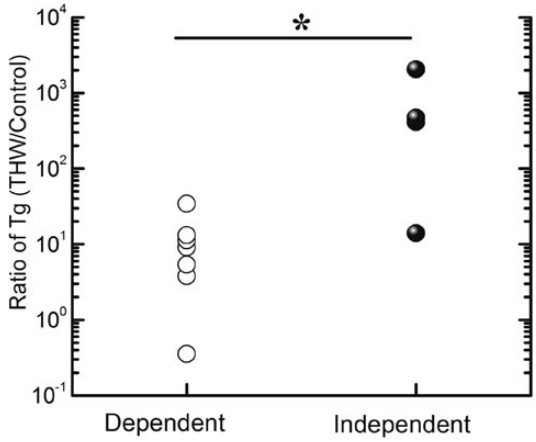

Figure 4. Ratio of Tg variation between radiation dose-dependent and dose-independent MN fractions. *$p < 0.05$.

signifies a normal response of thyroid function. In contrast, the radiation dose response during THW was modified so that saturation was >1.5 Gy (Figure 3). In addition, a higher concentration of Tg was observed (Figure 4).

This is the first study to show the specific dose response of MN frequency in association with thyroid-related hormones in cancer patients; however, these correlations have not been completely clarified. Several previous studies have described *in vitro* radiation dose–response curves calibrated by MN frequency in human lymphocytes, and many have reported an ionising radiation dose of ~4 Gy[1]. Recently, it has been clarified that internal radiation exposure to radioactive agents, such as ^{131}I and ^{18}F, affects MN frequency of PB lymphocytes and reticulocytes in cancer patients[6, 7]. The present study suggests that it is possible to identify the dose-dependent response of MN frequency in DTC patients on THW without the saturated frequency of more than 1.5 Gy.

It was considered that hormones, particularly Tg, may be the regulating factor for saturation. Thyroid-related hormones have a specific system consisting of TRH, TSH, Tg and fT4/fT3, wherein TSH and TRH are influenced by the negative-feedback function of fT4/fT3[5]. A decrease in TSH and an increase in fT3/fT4 in thyroid-ablated patients on DTC therapy were usually observed during thyroid hormone replacement therapy. Thus, the function of negative feedback in the present DTC patients was verified. In contrast, residual tumours and/or recurrent DTC often have up-regulated Tg concentrations, even when thyroid hormone replacement therapy is given. Cytokines (hormones) have the potential to induce cell proliferation, differentiation and inflammation by stimulating specific receptors. Tg is secreted from thyroid remnant tissue by stimulation of TSH[8]. Amakawa *et al.* reported that the levels of Tg mRNA in TSH-stimulated lymphocytes were noticeably increased in subjects with thyroid disease[9]. Therefore, this suggests that PB mononuclear cells may be directly or indirectly regulated by Tg.

Accumulation of information regarding factors that regulate MN frequency is required to estimate radiation dose in a population with individual variations in emergency radiation medicine. Thus, future studies should not only include healthy volunteers but also patients with underlying diseases. In addition, human haematopoietic cells have already been investigated because of higher radiosensitivity with individual variability[10].

In conclusion, this study clarified that a higher concentration of Tg under abnormal conditions modifies radiation dose response of MN frequency.

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