Radiosensitivity of Peripheral Blood Lymphocytes Obtained from Patients with Cancers of the Breast, Head and Neck or Cervix as Determined with a Micronucleus Assay

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Radiation sensitivity/Micronucleus assay/Cancer patients/Radiotherapy/Chemotherapy.

The in vitro radiation sensitivities of peripheral blood lymphocytes obtained from 48 normal females and 168 female cancer patients were measured with the cytokinesis-blocking micronucleus assay. Cancer patients group had significantly higher mean baseline micronucleus frequency than normal healthy controls. Breast cancer patients were more radiosensitive than normal individuals. Cervical cancer cases were less radiation sensitive than normal subjects. The relative lack of radiation sensitivity in cervical-cancer cases could be due to modification of the radiosensitivity of patients’ immune-responsible cells by human papillomaviruses infection. Normal individuals and cancer patients were classified according to their radiation sensitivity which was evaluated with the radiation-induced micronucleus frequencies. Such a classification will be an important initial step to characterize the radiosensitive, radioresistant, or cancer-prone individuals using specific SNP typing.

INTRODUCTION

Chromosomal damage and its repair assays such as the micronucleus (MN) assay¹ and G₂-phase assay² have been extensively used to evaluate the radiation sensitivity of human individuals.³⁵ Use of peripheral blood lymphocytes has become popular for quantifying human radiosensitivity, because of the ease of sample preparation. Scott et al demonstrated that a relatively large fraction of breast cancer patients had greater radiation sensitivity than normal, healthy women as assessed using the in vitro MN assay⁶⁻⁸ and the G₂-phase assay.⁷ Because Vral et al found that the intra-individual variability was not significantly different from the inter-individual variability for both the G₂-phase assay and the MN assay in their repeat experiments,⁹ they were skeptical of Scott et al’s findings. Vral et al suggested that the radiosensitivity of an individual should be determined with multiple measurements from multiple blood samples. We fully agree with their suggestion. However, for ethical reasons, it is very difficult or occasionally impossible to obtain multiple blood samples from a cancer patient. Then, we obtained one blood sample from each donor. Accordingly, the assay must provide good reproducibility. We already confirmed that the dose-MN induction assay had excellent reproducibility.³ The inter-individual variation was significantly larger than the intra-individual variation in Scott et al’s report⁷ and also in our previous report.¹⁰ Such a high radiation sensitivity determined with the MN assay or the G₂-phase assay using single blood sample from each donor has also been found in patients with head and neck cancer⁸ or brain tumor.¹⁰ If the cancer patients include a disproportionately large number of radiosensitive persons, some genes related to radiation sensitivity may be involved in tumorigenesis. To test this hypothesis in Japanese individuals, the in vitro radiation sensitivities of peripheral blood lymphocytes obtained from 48 normal females, 130 breast cancer patients, 7 head-and-neck cancer patients and 31 cervical cancer patients were measured with the cytokinesis-blocking micronucleus assay by comparing the X ray (2 Gy)-induced MN frequencies in cancer patients to those of volunteer controls. The association of radiotherapy or chemotherapy on baseline or induced MN frequency was also tested. This study demonstrated that the finding of Scott et al that a fraction of breast cancer patients had a higher radiation sensitivity than normal, healthy women was also

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observed in the Japanese. However, the radiation sensitivity of cervical cancer patients was lower than that of normal volunteers, though the number of cases was rather small. Our data demonstrate that radiotherapy slightly lowered the in vitro radiosensitivity of T-lymphocytes of cancer patients, but chemotherapy did not.

MATERIALS AND METHODS

Subjects

Blood samples were obtained from 48 normal healthy women (age range: 23–60 y; mean ± SD = 46.3 ± 9.8 y) recruited at the National Institute of Radiological Sciences (NIRS), Chiba and 130 breast-cancer (26–78 y; 53.2 ± 10.8 y), 7 head-and-neck cancer (51–69 y; 57.4 ± 5.8 y), and 31 cervical-cancer patients (36–80 y; 62.5 ± 12.9 y) diagnosed at the NIRS hospital or at the Chiba Cancer Center. To exclude the gender effect on baseline- and radiation-induced MN formation, all subjects were females. Smoking may be one of modifiers of the baseline- and radiation-induced MN frequencies. Less than 20% of subjects reported to be the current smokers. However, we could not obtain the accurate information of their smoking habit. Because our previous analysis found no significantly positive effect of smoking on background MN frequency and X-ray sensitivity as determined with cytokinesis-blocking micronucleus assay,3 we did not take the smoking habit into the analysis of this study. Informed consent was obtained from all blood donors. This study was approved by the Ethical Committee of NIRS.

Micronucleus assay

Blood samples containing sodium heparin as an anticoagulant were transported to laboratory at r.t. and cultured within 24 h of the blood being taken. A method previously reported2 was used for these experiments. Briefly, heparinized whole blood was diluted fivefold in RPMI medium (Sigma Chemical Co., St. Louis, MO) supplemented with 20% fetal calf serum (FCS; HyClone, Utah 84321, Lot No. AHJ9164). In all assays in this report, only one serum batch was used. The cell suspension was divided into aliquots, put into plastic test tubes (1.5 ml per tube (Beckton Dickinson Labware, Franklin Lakes, NJ; Cat. No. Falcon 35-2096), and irradiated with 0 or 2 Gy of X rays at room temperature. The X-ray generator (Shimadzu, Kyoto) was operated at 200 kVp, 20 mA, 0.5 mm Cu plus 0.5 mm Al external filter. Dose proximal to the cells was measured with a Victoreen densitometer. The dose-rate was 1.0 Gy/min. Cells were then cultured in the presence of Phytohaemagglutinin (PHA HA15; final concentration 10 µg/ml; Murex Biotech Limited, Dartford, UK; Cat. No. 30852701) at 37°C in a 95% air plus 5% CO₂ incubator for 42 h. Cultures were continued for another 24 h in the presence of cytochalasin B (final concentration 3 µg/ml: Sigma). Cells were washed once with Mg- and Ca-free PBS (PBS(−); Sigma) buffer, suspended into 5 ml of PBS(−) buffer, 5.5 ml of Carnoy’s fixative (methanol:glacial acetic acid 3:1, v/v) was added to the buffer saline, and the suspension was left standing for 15 min. The cells were fixed twice with Carnoy’s fixative to remove red blood cells. Drops of the cell suspension were then placed on glass slides and stained with 2% Giemsa (Murek, Darmstadt, Germany). MN in 500 binucleated cells for both dose levels within each donor were scored under a microscope (×1000) by each of two scorers. Criteria for scoring the MN were similar to those presented by Fenech and Morley [1]. If the difference between the two scorers’ values was larger than 15% of either value, additional 1000 cells were scored by both scorers.

Statistical analysis

Baseline MN frequency (the number of MN per binucleated cell) was analyzed using analysis of variance. The three cancer groups were compared to volunteers using Dunnett’s multiple comparison procedure. Comparisons among cancer sites or among chemotherapy types were made using Tukey’s multiple comparison procedure. Induced MN frequency was analyzed using a repeated-measures linear model with separate background parameters for each site of cancer and parameters for interactions between dose and site of cancer. The variance of MN frequency differed between 0 and 2 Gy in vitro frequencies, so the analyses were weighted by the inverse of the sample variance in each group defined by cancer site and in vitro dose. Plots of residuals were examined to verify the fits of all models and to confirm that equal variance was achieved with the weighted analysis. Transformation of the MN frequency was not necessary to achieve approximate normality for purposes of analysis. All analyses were performed using Minitab (State College, Pennsylvania).

RESULTS

Baseline frequency was not related to age at time of blood collection. The slope with age was −0.001 per 10 years (standard error 0.002, p = 0.63). All three cancer groups had significantly higher baseline MN frequency compared to normal individuals (p < 0.01); mean differences from the volunteers’ mean value [95% simultaneous confidence intervals] were: breast 0.008 [0.003, 0.012]; cervical 0.040 [0.034, 0.046]; head-and-neck 0.013 [0.003, 0.023]. Breast and head-and-neck cancer patients’ baseline frequencies were not significantly different from each other (p = 0.54), but were significantly higher than normal individuals (p < 0.02) and significantly lower than cervical cancer patients (p < 0.001). The reason for such strikingly high values in cervical-cancer patients (standard error 0.005, p < 0.001 compared to normal volunteers) was not clear.

Range and mean values of baseline and 2 Gy-induced MN

Range and mean values of baseline and 2 Gy-induced MN

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frequencies in the healthy controls and cancer patients are shown in Table 1. Figure 1 demonstrates the in vitro-induced MN frequencies in all individuals. The in vitro-induced MN frequency for normal individuals was 0.082 per Gy (standard error 0.004, p < 0.001). Those for patients with breast cancer or cervical cancer were significantly different from normal individuals in opposite directions (+0.021 per Gy for breast cancer patients, standard error 0.004; −0.038 per Gy for cervical cancer patients, standard error 0.005; p < 0.001). The in vitro-induced MN frequency for head-and-neck cancer patients was not significantly different from that of normal individuals (+0.015, standard error 0.010, p = 0.15), but given the small number of patients (7) it is difficult to conclude anything about the radiation sensitivity of these patients.

Data on radiotherapy and/or chemotherapy were available only for breast and cervical cancer patients. Three of the blood samples from breast-cancer patients were drawn while radiotherapy was in progress; these were combined with those whose blood was drawn after the completion of radiotherapy. Three breast-cancer cases with unknown radiotherapy status were excluded, leaving 92 patients (69 breast, 23 cervical) whose blood was drawn during or after 66 (58 breast, 8 cervical) prior to any radiotherapy (Table 2). The mean dose as of time of blood drawing was 51.9 Gy (standard deviation 6.4) in the breast-cancer patients and 53.8 Gy (standard deviation 9.1) in the cervical-cancer patients. As shown in Table 2, baseline MN frequency was suggestively higher in patients with prior radiotherapy by 0.003 (95% confidence interval [0, 0.007], p = 0.065). There was no indication of a difference in effect of radiation therapy on baseline MN frequency by type of cancer (p = 0.50). The greater proportion of cervical-cancer cases with prior radiation therapy did not explain the higher baseline MN frequency in that group. The induced MN frequency was, however, significantly related to radiotherapy (Table 2). The in vitro-induced MN frequency was lower in patients who received radiotherapy (p = 0.025). There was no indication of a difference in the variation of induced MN frequency following radiation therapy (p = 0.32), although the interquartile range, a more robust measure of spread given the presence of several potential outliers in the non-therapy group (Fig. 2), was slightly larger—0.110 versus 0.095.

Among the patients known to have received chemotherapy, 56 were sampled during the course of chemotherapy, six patients had blood sampled after the end of therapy, and there was one patient for whom it was not recorded whether the chemotherapy was still in progress. Because of small numbers, all of these patients were analyzed as a single group.

**Table 1.** Range of the baseline and the 2 Gy-induced MN frequencies in healthy women and cancer patients. Parenthesis means the mean value ± SD.

<table>
<thead>
<tr>
<th>Subjects (No. of individuals)</th>
<th>0 Gy</th>
<th>2 Gy</th>
<th>Range of the induced MN frequencies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy controls (48)</td>
<td>0.016 – 0.052 (0.031 ± 0.010)</td>
<td>0.070 – 0.428 (0.195 ± 0.068)</td>
<td>0.036 – 0.388 (0.163 ± 0.069)</td>
</tr>
<tr>
<td>Breast cancer patients (130)</td>
<td>0.017 – 0.078 (0.039 ± 0.009)</td>
<td>0.093 – 0.460 (0.244 ± 0.068)</td>
<td>0.065 – 0.419 (0.205 ± 0.069)</td>
</tr>
<tr>
<td>Cervical cancer patients (7)</td>
<td>0.032 – 0.102 (0.071 ± 0.016)</td>
<td>0.101 – 0.321 (0.158 ± 0.051)</td>
<td>0.028 – 0.243 (0.087 ± 0.050)</td>
</tr>
<tr>
<td>Head-and-neck cancer patients (31)</td>
<td>0.022 – 0.062 (0.044 ± 0.014)</td>
<td>0.154 – 0.342 (0.236 ± 0.065)</td>
<td>0.120 – 0.286 (0.192 ± 0.060)</td>
</tr>
</tbody>
</table>

**Fig. 1.** In vitro micronucleus frequency by cancer site. The lines connect the mean MN frequencies estimated by the repeated-measures model.

**Table 2.** Mean micronucleus frequency in in vitro irradiated or non-irradiated samples from breast or cervix patients with or without radiation therapy (standard error is indicated in parenthesis).

<table>
<thead>
<tr>
<th>Radiotherapy</th>
<th>Cancer site</th>
<th>Number of cases</th>
<th>In vitro radiation dose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 Gy</td>
<td>2 Gy</td>
</tr>
<tr>
<td>None as of time of blood sampling</td>
<td>Breast</td>
<td>58</td>
<td>0.037 (0.001)</td>
</tr>
<tr>
<td></td>
<td>Cervix</td>
<td>8</td>
<td>0.069 (0.003)</td>
</tr>
<tr>
<td>During or before time of blood sampling</td>
<td>Breast</td>
<td>69</td>
<td>0.040 (0.001)</td>
</tr>
<tr>
<td></td>
<td>Cervix</td>
<td>23</td>
<td>0.073 (0.004)</td>
</tr>
</tbody>
</table>
group. Only one cervical cancer patient had chemotherapy (her baseline MN frequency was typical but her induced frequency was large); she was excluded, leaving only breast cancer patients for the analyses of chemotherapy. For nine patients it was unknown whether they had chemotherapy. As their average baseline MN frequency was not intermediate to that for the other two groups (chemotherapy or not), they were also excluded. There was no evidence of an overall effect of chemotherapy (received or not) on baseline MN frequency (p = 0.46).

Chemotherapy was further divided into four groups based on classification of the compound(s) administered; group I: 35 patients received hormone derivatives (tamoxifen and its derivatives) only, group II: 14 patients received hormone derivatives + DNA metabolism inhibitors (5FU and its derivatives), group III: 2 patients received hormone derivatives and DNA metabolism inhibitors + DNA damaging agents (adriamycin, cyclophosphamide), and group IV: 3 patients received DNA metabolism inhibitors or DNA damaging agents alone (no hormone derivatives). There was a slight indication of increased baseline MN frequency in group III (Fig. 3), but it was not significant (p = 0.13, based on analysis of variance) given that the group included only two patients. The single high, outlying baseline MN frequency in group III is unlikely if all groups were equivalent (the probability that the maximum of 56 values from the same distribution is included by chance in a randomly sampled group of size 2 is 0.036). In terms of the in vitro induced MN frequency, there was no indication of an association with type of chemotherapy (analysis of variance p = 0.58), as revealed in the Fig. 4.

Although the three points from group IV were all below the overall average induced MN frequency, the probability of that occurring by chance is 0.125, which is not extremely unlikely.

DISCUSSION

The baseline MN frequencies in T-lymphocytes of
patients with breast cancer, head-and-neck cancer or cervical cancer were significantly higher than those of controls. Because it is widely believed that one key mechanism that leads to baseline micronucleus formation involves an imbalance of chromosomal segregation, chromosomal instability in these cancer patients' blood might be greater than that in normal individuals' blood. The reason for strikingly high values in cervical cancer patients was not clear. Cervical cancer patients in this study were not checked for infection with human papillomaviruses (HPVs). It is well known that more than 90% of cervical-cancer tissues contain HPV DNA. The intracellular signal network in lymphocytes as well as cervical epithelial cells might be disturbed by HPV infection. Such an altered intracellular signal network might enhance chromosomal instability.

The mean number of radiation-induced MN in breast cancer patients' cells was greater than that in normal females' cells. The radiosensitivity (in vitro-induced MN frequency) of breast cancer patients was significantly higher than that of normal healthy female (Fig. 1). A slope in head-and-neck cancer patients similar to that of breast-cancer patients was not significantly different from normal volunteers due to a small number of patients. Scott et al demonstrated that a relatively large fraction of breast cancer patients had greater radiation sensitivity than normal. Our study confirmed the Scott et al's finding in Japanese population. Our data obtained from Japanese breast-cancer patients therefore support the finding reported by Scott et al regarding English breast-cancer patients. On the contrary, the mean number of radiation-induced MN of cervical cancer patients was significantly lower than that of normal individuals (Fig. 1). Butz et al demonstrated that mitomycin C induces apoptosis in HPV DNA-positive cell lines. Harima et al found that the HPV DNA-negative patients with cervical carcinoma have a significantly poorer prognosis after radiotherapy than the virus DNA-positive cases. These reports suggest that infection with HPV could modify the radiosensitivity of infected patients' cells.

Clinical irradiation may have elevated the baseline MN frequency and significantly reduced the 2-Gy induced MN frequency (Table 2), although the increase in baseline frequency – about 5–10% - may be debatable. In our opinion, the difference is biologically meaningful, because it is about one-third of one standard deviation. Human lymphocytes exposed to low doses of ionizing radiations in vitro are insensitive to subsequent irradiations with large doses. This so-called ‘adaptive response in human lymphocytes’ has been reported in many in vitro studies of chromosome aberrations and micronucleus induction. Gil et al collected the blood samples from 11 thyroid cancer patients at one, six or 24 months after iodine-131 therapy. They treated the lymphocytes with mitomycin C (MMC) in vitro and scored the micronuclei. A significant decrease in MMC-induced micronucleus frequency occurred in cells obtained from 7 of 11 patients at one month after therapy. Although the blood collection in our study was not designed to investigate the in vivo adaptive response-like phenomenon, a small protective effect of prior clinical irradiations was suggested. On the contrary, hormone derivatives (tamoxifen and its derivatives) and DNA metabolism inhibitors (5FU and its derivatives) did not modify the radiosensitivity. Although the numbers of patients in this study who received DNA-damaging agents were too small to allow thorough statistical analysis, calculation of simple probabilities did not suggest that the DNA damage induced by adriamycin or cyclophosphamide was involved in the in vivo adaptive response (Fig. 3).

Polymorphisms in DNA repair genes have been extensively studied in regards to cancer risk. Micronuclei occur as a consequence of DNA double-strand breaks. It is plausible that DNA repair-related genes with low penetration are involved in the enhanced radiation susceptibility to radiation-induced micronuclei of T-lymphocytes from patients with breast or other cancers. XRCC2 and XRCC3 are the key proteins in the homologous recombination repair pathway, which repairs DNA double-strand breaks (DSBs). Recently, Kuschel et al demonstrated that ratios of two single nucleotide polymorphisms (SNPs; 17893A/G and 18067C/T) in XRCC3 were significantly different between cancer patients and normal females. Interestingly, 18067C/T has been demonstrated to be associated with risk of malignant melanoma and squamous cell carcinoma of the head-or-neck. Rafii et al demonstrated that the 31479G/A polymorphism in XRCC2 was a low-penetrating susceptibility factor for breast cancer. However, Rodriguez-Lopez et al failed to detect mutations in the XRCC2 in 105 Spanish high-risk breast/ovarian cancer families. These SNPs data suggest that DSB repair-related genes with low penetration may be involved in tumorigenesis and also played a role in patients' radiation sensitivity.

We are planning to further characterize the radiosensitive, radioresistant-, or cancer-prone-individuals using specific SNP typing. The micronucleus assay may be a useful strategy for the initial classification of normal individuals and cancer patients according to their radiation sensitivity.

ACKNOWLEDGEMENT

The authors thank to Mses. H. Tanaka and H. Sudo for their technical help, Dr. H. Toyama, Ms. Y. Matsuda of Medical Information Processing Office, Research Center for Charged Particle Therapy, NIRS for supervising the anonymity system, Drs. J. Mizoe, T. Yanagi, N. Yamamoto and Misses T. Satoh, R. Haneishi, S. Tsuruoka, S. Shimoda and S. Kanazawa of Research Center Hospital, NIRS for their continuous support of Dr. Hirohiko Tsuji, the Chairman of Research Center for Charged Particle Therapy, and Dr. Hajime Mura-
ta, the Chairman of Frontier Research Center, is grateful acknowledged.

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