Proliferative Activity and Micronucleus Frequency after Radiation of Lung Cancer Cells as Assessed by the Cytokinesis-Block Method and Their Relationship to Clinical Outcome

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ABSTRACT
We previously proposed a new assay using the cytokinesis-block micronucleus (MN) technique to estimate the fraction of cells undergoing mitosis in vitro [dividing fraction (DF)], potential doubling time (T_{pot}), and radiosensitivity (in terms of MN frequency) of human tumors. In the present study, we applied this technique to primary lung cancers to evaluate their biological characteristics, and the assay results for the proliferative activity were compared with the treatment outcome. Tumor tissues were disaggregated to single cells, which were cultured in the presence of cytochalasin B after (or without) radiation. At intervals, the proportion of multinucleate cells (its maximum value is the DF), the average number of nuclei/cell, and MNs in binucleate cells were scored. The T_{pot} was the extrapolated time for the nucleus:cell ratio to reach 2.0. Of the 71 tumor samples obtained, the DF and T_{pot} were evaluable in 61 (86%), and the MN frequency was evaluable in 52 (73%). The median DF and T_{pot} values were 23% and 7.7 days, respectively, for adenocarcinoma (n = 41), 26% and 8.9 days for squamous cell carcinoma (n = 13), 27% and 6.5 days for large cell carcinoma (n = 3), and 30% and 7.0 days for small cell carcinoma (n = 4). There was no significant difference in the mean DF or T_{pot} values according to the histological type or disease stage. The mean MN frequency after 2 Gy of radiation (minus the 0 Gy frequency) was 0.15 for adenocarcinoma, 0.17 for squamous cell carcinoma, 0.16 for large cell carcinoma, and 0.20 for small cell carcinoma. The MN frequency after radiation was positively correlated with both the DF and the baseline (at 0 Gy) MN frequency. In non-small cell lung cancer, a DF above the median was associated with an increased recurrence rate after operation, and the T_{pot} was correlated with the time until relapse in patients who developed recurrence. Although the clinical significance of the MN frequency needs to be clarified in future studies, the DF and T_{pot} determined by this assay appear to be good parameters of tumor proliferative activity.

INTRODUCTION
Human tumors vary in their biological characteristics and responses to antitumor treatments. Hence, optimization of therapy based on the predicted characteristics of each tumor is expected to produce better treatment outcome. In radiation therapy, the intrinsic radiosensitivity, proliferative activity (especially the T_{pot}^2), and oxygenation status of tumor cells are considered to be three major determinants of responsiveness to radiation. Each of these parameters can now be measured by various approaches. For example, the intrinsic radiosensitivity can be measured using the cell survival assay (1, 2), the T_{pot} using the bromodeoxyuridine or iododeoxyuridine-flow cytometry method (3, 4), and tumor hypoxia using the polarographic electrode system (5, 6). However, these methods are quite different from each other, and to evaluate the overall radiosensitivity of tumors, these different assays or measurements must be performed separately.

In our previous studies, we devised a method of simultaneously estimating two of these three parameters (radiosensitivity and proliferative activity, including T_{pot}^2) in human tumors (7–9). This assay uses the cytokinesis-block MN method and involves the determination of MN frequency after radiation, the fraction of tumor cells undergoing mitosis in vitro (the DF), and the extrapolated time for the tumor cell nuclei to double in culture (in vitro T_{pot}). The usefulness of this assay has recently been corroborated by other investigators (10), and this assay is now being increasingly used in studies of human and rodent tumors, in part as an alternative to the flow cytometry method of estimating T_{pot} (9–11).

In a previous study, we established this assay in human tumor cells in primary culture and demonstrated its feasibility in various types of human tumors (9). In the present study, we used this assay to characterize primary lung cancers of different histologies and stages with respect to the proliferative activity and the pattern of MN production by radiation, and we investigated the relationship of the proliferative activity data with the clinical outcome. We did not assess the correlation between MN frequency and radiation response, because the majority of the patients did not undergo radiotherapy.

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2 The abbreviations used are: T_{pot}, potential doubling time; MN, micronucleus; DF, dividing fraction; BNC, binucleate cell; MNC, multinucleate cell; PCNA, proliferating cell nuclear antigen.
MATERIALS AND METHODS

Assay Procedure. All of the specimens were obtained at thoracotomy and not by bronchoscopic biopsy. A total of 71 primary lung cancer specimens obtained from patients without preoperative radiation or chemotherapy were evaluated. The weights of the tumors obtained ranged from 54 mg to 6.0 g, with a median of 1.9 g. The assay method has been described in detail previously (9). Briefly, tumor specimens were minced with scissors and treated at 37°C for 2 h with 1 mg/ml collagenase/dispase (Boehringer Mannheim, Mannheim, Germany) dissolved in PBS. Then the resulting tumor cell suspension was filtered, and viable cells were counted using trypan blue. After removal of the collagenase/dispase solution by centrifugation, the cells were plated onto multiple collagen-coated dishes (20 cm², Iwaki Glass, Tokyo, Japan). Whenever the cell yield was sufficient, 3–6 × 10⁵ cells/dish were plated onto 10 dishes. The culture medium used was Ham’s F-12 supplemented with 20% fetal bovine serum and 0.2 mg/ml gentamicin sulfate.

Within 1 h after plating, 2 or 4 Gy of radiation was given to some of the dishes (usually two dishes per dose), using a cobalt-60 source in most cases. Within 30 min after irradiation, cytochalasin B dissolved in DMSO was added to all dishes at a concentration of 1.5 μg/ml. This concentration of cytochalasin B appeared to be optimal in all of the human tumor cells tested in the previous study (9).

Cultures were terminated at various intervals, and the cells were fixed with 1% glutaraldehyde in phosphate buffer, treated with 5 N hydrochloric acid for 20 min, and stained in the dark with Schiff’s reagent for 1 h as described previously (7). Usually unirradiated cells were fixed on days 1, 2, 3, 4, 6, and 8. By monitoring the increase in the number of BNCs in the unirradiated dishes, the optimal days for fixing the irradiated cells were determined; they were usually days 4–6.

Scoring and Analysis. Tumor cells were distinguished from normal cells on the basis of morphological criteria such as nuclear irregularity, dense nuclear staining, and a high nucleocytoplasmic ratio (12), and only those distinguished as tumor cells were scored. The cells with different numbers of nuclei (mononucleate, binucleate, trinucleate, and so forth) and the MNCs in the BNCs were counted under a microscope at a magnification of 1000. At least 100 cells (250–300 whenever possible) were assessed per dish, and at least 50 BNCs (100–150 whenever possible) were assessed to determine the MN frequency. When these numbers of cells could not be assessed, the assay was regarded as unsuccessful. BNCs with three or more MNS were occasionally found, but all MNS were scored.

Then, the percentage of MNCs (cells with two or more nuclei), the average number of nuclei/cell, and the average number of MNS per single BNC (MN frequency) were calculated. The DF (maximal MNC percentage) and Tpot were estimated from the unirradiated group of cultures as described previously (8, 9). The Tpot obtained with this assay was the extrapolated time for the nuclear ratio (the average number of nuclei per tumor cell) to reach 2.0. When the MN frequencies at different culture times (after day 3) were not significantly different from each other, the mean of these values was taken as the representative MN frequency for both unirradiated and irradiated cells.

RESULTS

Patient Staging, Follow-up, and Statistical Analysis. All patients were staged preoperatively according to the 1992 tumor-node-metastasis (TNM) system, using chest, brain, and abdominal computed tomography and bone and gallium-67 scintigraphy. After operation, pathological staging was used to indicate the disease stage. Postoperatively, oral UFT (tegafur + uracil) was used in patients at ≥ stage II, but systemic cisplatin-based chemotherapy was only used for stage IV or small cell lung cancer patients. Only three patients received postoperative radiotherapy. The median and minimum follow-up periods were 23 and 6 months, respectively, for the living patients. Relapse-free survival rates were calculated from the day of operation using the Kaplan-Meier method, and differences in the rate were examined by the log-rank test. The interactive effect of potential prognostic factors on the relapse-free survival was examined using the Cox proportional hazards model. Differences in the mean values of pairs of the data were examined using Student’s or Welch’s t test.

Assay Data. Fig. 1 shows a representative assay result for an adenocarcinoma. As shown, three sets of data (i.e., DF, Tpot, and MN frequency) were obtained with this assay. The proportion of MNCs appeared to reach a plateau within 4–6 days in all tumor cells investigated, and the DF was defined as the mean of the percentage of MNCs at the plateau. Tpot was obtained by fitting the initial part (for days 1–3) of the nuclear ratio curve to an exponential curve and extrapolating from it, as shown in Fig. 1, when necessary. This extrapolation was necessary in all but one of the tumors in which the nuclear ratio did not reach 2.0.

Of the 71 tumors tested, the DF and Tpot were obtained in 61 (86%) and the MN frequency was evaluable in 52 (73%). The assay success rates for DF/Tpot and MN frequency were 91 and 78%, respectively, for adenocarcinoma, 81 and 69% for squamous cell carcinoma, 75 and 75% for large cell carcinoma, and 67 and 50% for small cell carcinoma.
Table 1  Assay data according to the histological type of lung cancer

<table>
<thead>
<tr>
<th>Histology</th>
<th>n</th>
<th>Mean ± SD (Median [range])</th>
<th>Mean ± SD (Median [range])</th>
<th>n</th>
<th>0 Gy</th>
<th>2 Gy</th>
<th>4 Gy</th>
<th>2 Gy - 0 Gy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenocarcinoma</td>
<td>41</td>
<td>23 ± 9 (23.0-45)</td>
<td>9.6 ± 5.2 (4.2-31)</td>
<td>41</td>
<td>0.11±0.05</td>
<td>0.26±0.12</td>
<td>0.36±0.18</td>
<td>0.15±0.07</td>
</tr>
<tr>
<td>Squamous cell cancer</td>
<td>13</td>
<td>26 ± 11 (26.1-53)</td>
<td>6.9 ± 4.1 (4.5-19)</td>
<td>13</td>
<td>0.10±0.03</td>
<td>0.27±0.06</td>
<td>0.37±0.11</td>
<td>0.17±0.05</td>
</tr>
<tr>
<td>Large cell carcinoma</td>
<td>3</td>
<td>28 ± 4 (27.25-32)</td>
<td>7.2 ± 1.5 (6.2-9.0)</td>
<td>3</td>
<td>0.11±0.04</td>
<td>0.27±0.07</td>
<td>0.41±0.08</td>
<td>0.16±0.03</td>
</tr>
<tr>
<td>Small cell carcinoma</td>
<td>4</td>
<td>30 ± 4 (25-35)</td>
<td>6.8 ± 2.8 (3.1-10)</td>
<td>4</td>
<td>0.11±0.05</td>
<td>0.32±0.08</td>
<td>0.47±0.16</td>
<td>0.20±0.04</td>
</tr>
</tbody>
</table>

For all tumors assessed, the DF value ranged between 9.0 and 53%, with a median of 25%, and the $T_{pot}$ ranged between 3.1 and 31 days, with a median of 8.2 days. There was a correlation between the DF and $T_{pot}$ ($r = -0.73; P = 0.0000$). The median (range) for the MN frequency at 0 Gy, 2 Gy, and 4 Gy levels was 0.10 (0.041–0.25), 0.25 (0.12–0.58), and 0.33 (0.17–1.0), respectively. Table 1 shows the assay data according to the histological type of lung cancers. Although the numbers of evaluable specimens were small for large cell and small cell carcinomas, there appeared to be no difference in any of the DF, $T_{pot}$, and MN frequency due to the histology. There was no difference between adenocarcinoma and squamous cell carcinoma. Table 2 shows the assay data according to the disease stage. Although there appeared to be a weak trend toward tumors at higher stages having higher DF and shorter $T_{pot}$, the difference was not significant between any of the stages. The MN frequency also did not seem to differ by stage.

Correlation of DF and $T_{pot}$ with Clinical Outcome. The patients with non-small cell lung cancer at each disease stage were divided into two groups according to the DF value ($\geq$ or <median of each stage). Fig. 2 shows postoperative relapse-free survival curves for the patients with stage I-IIIB non-small cell cancer according to the DF value. The 28 patients with DF $\geq$median for each stage had significantly poorer relapse-free survival rate than the 27 patients with DF below the median ($P = 0.0069$). Table 3 shows the result of multivariate analysis of potential prognostic factors including age, sex, histology, stage, and DF value. Among them, stage and DF were found to significantly influence relapse-free survival. The significant effect of DF was also observed even when data for two patients with non-stage IV, small cell lung cancer were included in the analysis (data not shown).

Twenty-one patients with stage I-IIIB disease who underwent macroscopic curative surgery developed recurrence. In these patients, a correlation was found between the time to recurrence and their $T_{pot}$ value (Fig. 3). On average, recurrence was detected after 12 months in patients with a $T_{pot}$ of 8 days.

Pattern of MN Induction. The MN frequency at 0 and 2 Gy was evaluable in 52 patients, but in 3 of them, the frequency at 4 Gy could not be obtained because of the relatively small cell yield or culture contamination. Thus, the dose-MN frequency curve (from 0 to 4 Gy) was evaluable in 49 patients. The curve could be fitted with a straight line ($r \approx 0.997$) in 22 patients (45%) but not in the other 27 patients (55%). In all of the latter patients, the curve was convex, and in none of the patients was it exponential. There was a correlation between the MN frequency at 2 or 4 Gy with subtraction of the 0 Gy frequency and the DF/$T_{pot}$. The higher MN frequency after radiation was more often seen in the tumors with higher DF (Fig. 4) or shorter $T_{pot}$ (data not shown). There was also a weak correlation between the DF and MN frequency at 0 Gy ($r = 0.30; P = 0.029$; data not shown). In addition, there was a strong correlation between the MN frequency at 0 Gy and that at 2 Gy (Fig. 5), that at 4 Gy, or that at 2 Gy minus the 0 Gy value (data not shown); tumors with higher spontaneous MN frequency tended to produce more MNs following radiation.

DISCUSSION

In this study, we successfully applied the cytokinesis-block assay to primary lung cancers and obtained three sets of data on the DF, $T_{pot}$, and MN frequency. This assay is useful not only because it provides information on both proliferative activity and radiosensitivity but also because the data may be obtained in only a week. Moreover, this assay has the additional advantage that normal cells can be excluded from scoring. The assay method usually requires at least 100 mg of tumor tissue. To make this assay applicable to relatively small biopsy specimens, minor improvement of the method, including the use of other...
enzyme cocktails to obtain higher cell yield and the use of smaller culture dishes with a special device for microscopic observation, seems to be necessary.

There appeared to be no differences among non-small cell carcinomas in the average values of DF and $T_{pot}$, although the number of large cell carcinomas was small. These results are consistent with those of other studies demonstrating no differences among the three subtypes of lung cancer in the positivity rate for various proliferation markers (13, 14), and they would provide a rationale for grouping these three carcinomas as non-small cell lung cancer. A weak trend could be seen for stage I–II tumors to have lower DF and higher $T_{pot}$ values than stage III–IV tumors, but the difference was not significant. Our results seem to be intermediate between the two opposing sets of reported results, one showing no difference in proliferative activity due to stage (13) and another showing higher proliferative activity in higher stage lung cancers (14, 15).

There was also no difference in the MN frequency with or without irradiation between adenocarcinoma and squamous cell carcinoma. This is consistent with the data on uterine carcinoma showing similar values for the surviving fraction at 2 Gy in the two types of carcinomas (16, 17). Although squamous cell carcinomas regress at an earlier time than do adenocarcinomas, the overall prognosis after radiotherapy is similar in patients with adenocarcinoma and those with squamous cell carcinoma of the lung (18, 19). Our data would support this clinical observation. Small cell carcinomas are much more radiosensitive than non-small cell carcinomas, but in this study, although the number of small cell carcinomas was small, the MN frequency after radiation for these tumors was similar to or only slightly higher than that for non-small cell carcinomas. Small cell carcinomas appear to die more often by apoptosis (20), and apoptosis and MN-related death are different events (21). This may explain our observation.

In all tumor cell cultures, the proportion of MNCs did not continue to increase with culture time and appeared to reach a plateau within 4–6 days. This is consistent with our previous findings obtained with murine, xenografted human, and various other human tumors (8, 9). Therefore, we could define the DF as the maximum proportion of MNCs. With respect to the prognostic value of the DF, we previously observed that a DF slightly higher than that for non-small cell carcinomas. Small cell carcinomas appear to die more often by apoptosis (20), and apoptosis and MN-related death are different events (21). This may explain our observation.

In all tumor cell cultures, the proportion of MNCs did not continue to increase with culture time and appeared to reach a plateau within 4–6 days. This is consistent with our previous findings obtained with murine, xenografted human, and various other human tumors (8, 9). Therefore, we could define the DF as the maximum proportion of MNCs. With respect to the prognostic value of the DF, we previously observed that a DF slightly higher than that for non-small cell carcinomas, was associated with a higher recurrence rate in a very heterogeneous group of tumors (9). In this study, we confirmed this finding in a much more homogenous group of non-small cell lung cancer patients, as well as in patients with lung cancer of all types. Therefore, as well as other proliferation markers such as PCNA and Ki-67, the DF seems to be a useful indicator of tumor proliferative activity and patient prognosis. The relationship between the DF and the growth fraction as determined by PCNA, Ki-67, or other proliferative markers is of interest, because the median DF value of 25% in our study does not differ significantly from the reported Ki-67 or PCNA positivity rates of 18–38% for non-small cell lung cancer (13, 14). We have just started to investigate whether and to what extent the MNCs correspond to the proliferation marker-positive cells.

The $T_{pot}$ is now considered an important parameter to select patients for accelerated treatment. The flow cytometry method is now being widely used to estimate the $T_{pot}$. However, several methodological problems that make the obtained $T_{pot}$ value inaccurate have been pointed out, including the influence of normal cell counts in diploid tumors and interlaboratory variations (22–24). Indeed, some recent studies suggest that the $T_{pot}$ obtained by the flow cytometry method is not necessarily.

**Table 3** Multivariate analysis of potential prognostic factors in stage I–IIIB non-small cell lung cancer patients

<table>
<thead>
<tr>
<th>Variable</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>0.98</td>
</tr>
<tr>
<td>Age (&lt; or $\geq$ 65 years)</td>
<td>0.84</td>
</tr>
<tr>
<td>Histology</td>
<td>0.66</td>
</tr>
<tr>
<td>Stage</td>
<td>0.00018</td>
</tr>
<tr>
<td>Dividing fraction (&lt; or $\geq$ median for each stage)</td>
<td>0.010</td>
</tr>
</tbody>
</table>

$Fig. 2$ Relationship between the DF and time to recurrence in patients with stage I–IIIB non-small cell lung cancer. $r = 0.70; P = 0.00044$. 

$Fig. 3$ Correlation between the $T_{pot}$ and time to recurrence in 21 patients with stage I–IIIB lung cancer. $r = 0.70; P = 0.00044$. 

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useful in the clinic (25). As an alternative, our method appears to be attracting attention recently. There are only a few data on the $T_{pot}$ values of lung cancer measured by the flow cytometry method, but two studies both demonstrated the median values of 7.0–7.1 days (26, 27), which almost accord with our data. However, these median $T_{pot}$ values are apparently longer than those reported for head and neck cancers (4.6–5 days; Refs. 3, 4, and 25), uterine cervical cancers (5.5 days; Ref. 24), and rectal cancers (3.3 days; Ref. 28), although they are shorter than that for soft tissue sarcomas (11.7 days; Ref. 29). Our $T_{pot}$ measurement is based on the kinetics of nuclear division in vitro, and it is not surprising that the $T_{pot}$ measured by our method tends to be an overestimate. Whatever the true $T_{pot}$ value is, however, we think our method is sufficiently accurate to rank tumors according to the proliferative rate. The $T_{pot}$ is known to represent the proliferation rate of tumors recurring after radiotherapy better than the volume doubling time does (30, 31). We are not sure whether or not the regrowth rate of tumors after surgery is related to the $T_{pot}$, but we found a positive correlation between the $T_{pot}$ and the time until recurrence. Thus, the $T_{pot}$ may also be useful in predicting the postoperative period at high risk for recurrence.

Whether the MN frequency after radiation represents the radiosensitivity is a matter of controversy. Some authors found a good correlation between the MN frequency and cell survival (7, 32), whereas others found no such correlation (33, 34). Because the cell survival is not necessarily an absolute measure of radiosensitivity, such clinical studies comparing the MN frequency with actual tumor response are advisable. We could not investigate this issue in the current study because the primary tumor was totally resected in all patients, but such a study is ongoing with brain tumors to which radiation is often applied after partial tumor resection.

Although we did not examine the usefulness of the MN assay as a radiosensitivity test, a number of findings were obtained regarding the MN production in lung cancer cells. First, the MN frequency after radiation was correlated with the DF. This finding seems to be consistent with the classic Bergonie-Tribondeau law, which states that cells with higher proliferative activity are likely to be more radiosensitive. Second, the MN frequency after radiation was closely correlated with the baseline frequency (at 0 Gy). This observation calls into question whether the index MN/BNC (2 Gy – 0 Gy) is the most appropriate or not. Different indices such as MN/BNC (2 Gy/0 Gy) or MN/BNC [(2 Gy – 0 Gy)/0 Gy] should also be investigated in future studies. Third, the MN frequency did not linearly or exponentially increase at 4 Gy as compared with the values at 0 and 2 Gy in more than half of the tumors. This phenomenon has already been reported in established murine and human cell lines by Abend et al. (21), who attributed it to the development of apoptosis at higher doses. In future studies, therefore, it may be worthwhile to modify the method to include scoring of both micronucleated cells and apoptotic cells. Thus, MN frequency determination should be further optimized to use the MN frequency as a measure of radiosensitivity.

In summary, the cytokinesis-block assay is feasible in human lung cancer cells in primary culture. This assay provides three sets of data, for DF, $T_{pot}$, and MN frequency. The DF appears to be an index of tumor proliferative activity, and the $T_{pot}$ obtained with this method was correlated with the time until recurrence. Whether or not the MN frequency after 2 Gy of radiation represents clinical radiosensitivity of the tumor is a topic of future investigation.

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Proliferative activity and micronucleus frequency after radiation of lung cancer cells as assessed by the cytokinesis-block method and their relationship to clinical outcome.

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