Radiation Sensitivity in Vitro of Primary Tumors and Metastatic Lesions of Malignant Melanoma

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ABSTRACT

The radiation sensitivity of cells isolated directly from surgical specimens of the primary tumor and one to three distant metastases in ten different melanoma patients was measured in vitro using the Courtenay agar colony assay. Dose-response curves were fitted to the cell survival data by the method of least squares using the multitarget-single hit and the linear-quadratic models. The ten patients could be divided into three distinct groups. Group I consisted of four patients with radiosensitive primary tumors (\(D_0\) from 1.38 ± 0.06 Gy to 1.69 ± 0.08 Gy). The radiation sensitivity of the metastases (\(D_0\) from 1.33 ± 0.10 Gy to 1.73 ± 0.06 Gy) was not significantly different from that of the primary tumor in this group; i.e., no heterogeneity was observed. Group II consisted of three patients with radioresistant primary tumors (\(D_0\) from 0.84 ± 0.06 Gy to 0.91 ± 0.05 Gy). Heterogeneity was not observed in this group either; i.e., all metastases were radiosensitive (\(D_0\) from 0.85 ± 0.05 Gy to 1.00 ± 0.06 Gy). Group III consisted of three patients with a heterogeneous disease. The primary tumor of all patients in this group was radiosensitive (\(D_0\) from 0.85 ± 0.05 Gy to 1.03 ± 0.05 Gy). The most radioresistant metastasis in each patient was significantly (\(P < 0.05\)) more resistant (\(D_0\) from 1.46 ± 0.06 Gy to 1.56 ± 0.07 Gy) than the primary tumor. None of the metastases were significantly more radiosensitive than the primary tumor in any patient. These observations suggest that the progression of tumors to increased levels of malignancy includes the increased ability to become radioresistant, and it may be speculated that similar genomic alterations are responsible for the development of the metastatic and the radioresistant tumor cell phenotype. If so, this may have severe implications for the treatment of metastatic malignant melanoma with low linear energy transfer ionizing radiation as well as for the development of predictive assays for tumor treatment sensitivity.

INTRODUCTION

Malignant tumors develop clonal heterogeneity during growth and therapeutic intervention; i.e., they often consist of cell subpopulations possessing differential phenotypic characteristics, including metastatic potential and sensitivity to treatment (1, 2). The cellular composition of the primary tumor and metastatic lesions in the same patient is therefore frequently disparate (3). The mechanism by which clonal heterogeneity originates is not clear. Different genomic alterations such as point mutations, deletions, and gene amplification can be involved (4, 5). Genomic instability is a characteristic feature of tumor cells (6, 7), and it has been suggested that this instability is caused by errors in tumor suppressor genes, leading to clonal tumor heterogeneity (3, 8).

There is some evidence that similar underlying mechanisms are involved in the development of increased metastatic potential and drug resistance (9). This view is supported by clinical studies which have demonstrated clear differences in sensitivity to chemotherapy between primary tumors and metastatic deposits for many cancer types (10). Moreover, different chemosensitivity profiles have been demonstrated in vitro for primary tumors and their metastases and for individual metastases in the same patient, by using a human tumor colony-forming assay (11–13).

Clonal heterogeneity in radiation sensitivity has been demonstrated for human tumor cell lines (14, 15) and human tumors heterotransplanted into athymic mice (16). Studies comparing the cellular radiation sensitivity of different lesions in the same patient are sparse (17). The radiation sensitivity in vitro of cells isolated from surgical specimens of the primary tumor and one to three distant metastases in ten different melanoma patients is reported in this paper. The main purpose of the work was to shed light on a possible parallelism in the development of metastatic cell populations and the development of cellular radiation resistance in tumors. The work was also designed to elucidate the potential usefulness of ionizing radiation in the treatment of tumor metastases. Malignant melanoma was chosen for this study because this disease develops in a sequence of steps, progressing from benign proliferative tumors, to primary tumors that do not show evidence of metastases, to invasive primary tumors, and finally to regional and distant metastases (18). Clinical, histopathological, immunological, and genetic criteria have been established to distinguish the different steps in the progression of melanoma (18–20). Also, the cellular radiation sensitivity of melanoma differs considerably among individual patients; \(D_0\) ranging from 0.57 Gy to 2.11 Gy have been reported (21). Moreover, cells isolated from human melanoma surgical specimens usually show a higher PE in vitro than do cells isolated from other tumor types (22, 23).

MATERIALS AND METHODS

Tumor Specimens. Tumor tissue specimens from melanoma patients were dropped into culture medium at 4°C immediately after surgery and brought to the laboratory. Normal tissue, necrotic areas, and blood clots were removed, using a scalpel and a pair of tweezers. The remaining tumor tissue was rinsed several times in culture medium and cut into small fragments. A standardized mechanical procedure was used to prepare single cell suspensions from the fragments. They were put into a plastic bag with 20 ml of culture medium and disaggregated for 30 s with a stomacher (Lab-Blender 80). The resulting suspensions were filtered through 30-μm nylon mesh to remove cell aggregates, centrifuged, and resuspended in culture medium. The quality of the suspensions was examined, using a phase-contrast microscope and a hemocytometer. The fraction of doublers was always <5%, and the fraction of larger aggregates was always <0.1%. Morphologically intact, metabolically viable cells, i.e., cells having an intact and smooth outline with a bright halo, were counted. The cell suspensions were then diluted to appropriate concentrations and used in radiation experiments. When the cell yield was high, some of the cells were frozen and stored in liquid nitrogen for use in experiments at a later time.

Tissue specimens from the primary tumor and the metastases of a given patient were obtained on the same day with one exception; the two lymph node metastases of Patient V. B. were removed surgically 6
treated with 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide and
mo after the primary tumor and the s.c. metastasis. This patient was
exposure.

ture at a dose rate of 3.4 Gy/min. The cells were kept in 1 ml of soft agar
20 mA, and with 0.5-mm Cu filtration, was used for irradiation. The
clonogenic. PE was calculated from the number of colonies counted and
O2, 5% CO2, and 90% N2 was used to flush the tubes before irradiation. The melanoma cells were incubated at 37°C in an atmosphere of 5%
Erythrocytes from August rats were added before melanoma cells were
seeded in plastic tubes (Falcon No. 2057). The tubes were plunged into
4 to 5 wk after treatment. Culture medium
from human tumors (22, 23). Care was taken to avoid these pitfalls, (a)
pecking was performed (26).

**RESULTS**

Radiation cell survival curves were successfully established from the primary tumor and one to three distant metastases in ten different patients. The PE of the metastases was always somewhat higher than or similar to that of the corresponding primary tumor. All specimens showed a sufficiently high PE that cell survival could be measured over two to three decades. The cell yield for most of the specimens was high enough that the experiments could be repeated with cell samples stored in liquid nitrogen. Experiments with stored cells gave similar results to experiments with newly prepared cell suspensions, in agreement with previous experience (22, 23). The cell survival curves are illustrated in Figs. 1 to 3, and the cell survival curve parameters and the PEs are presented in Table 1 together with the tumor locations and the patients’ sex and age.

The ten patients were divided into three groups on the basis of the cellular radiation sensitivity of their tumors (Table 1).

**Table 1** Radiation survival curve parameters

<table>
<thead>
<tr>
<th>Patient</th>
<th>Tumor</th>
<th>Sex</th>
<th>Age (yr)</th>
<th>Site</th>
<th>$D_{0}$ (Gy)</th>
<th>$n$</th>
<th>$\alpha$ (Gy$^{-1}$)</th>
<th>$\beta$ (Gy$^{-2}$)</th>
<th>$SF_{2}$</th>
<th>PE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F. S.</td>
<td>P</td>
<td>F</td>
<td>30</td>
<td>Lower leg</td>
<td>1.45 ± 0.08</td>
<td>1.7 ± 0.3</td>
<td>0.49 ± 0.06</td>
<td>0.018 ± 0.007</td>
<td>0.43$^a$</td>
<td>2.4</td>
</tr>
<tr>
<td>F. S.</td>
<td>M1</td>
<td>F</td>
<td>30</td>
<td>Lymph node</td>
<td>1.46 ± 0.06</td>
<td>1.9 ± 0.3</td>
<td>0.43 ± 0.05</td>
<td>0.024 ± 0.006</td>
<td>0.48</td>
<td>5.1</td>
</tr>
<tr>
<td>F. S.</td>
<td>M2</td>
<td>F</td>
<td>30</td>
<td>Lymph node</td>
<td>1.45 ± 0.06</td>
<td>1.4 ± 0.3</td>
<td>0.54 ± 0.04</td>
<td>0.013 ± 0.005</td>
<td>0.36</td>
<td>4.0</td>
</tr>
<tr>
<td>N. A. M</td>
<td>P</td>
<td>M</td>
<td>36</td>
<td>Scalp</td>
<td>1.45 ± 0.12</td>
<td>2.1 ± 1.0</td>
<td>0.37 ± 0.08</td>
<td>0.023 ± 0.009</td>
<td>0.65</td>
<td>7.7</td>
</tr>
<tr>
<td>N. A. M</td>
<td>M</td>
<td>M</td>
<td>36</td>
<td>Lymph node</td>
<td>1.54 ± 0.06</td>
<td>2.4 ± 0.4</td>
<td>0.31 ± 0.06</td>
<td>0.027 ± 0.006</td>
<td>0.64</td>
<td>6.9</td>
</tr>
<tr>
<td>S. D.</td>
<td>P</td>
<td>M</td>
<td>51</td>
<td>Heel</td>
<td>1.69 ± 0.08</td>
<td>1.9 ± 0.4</td>
<td>0.37 ± 0.05</td>
<td>0.016 ± 0.005</td>
<td>0.60</td>
<td>3.5</td>
</tr>
<tr>
<td>S. D.</td>
<td>M1</td>
<td>M</td>
<td>51</td>
<td>s.c. tissue</td>
<td>1.73 ± 0.06</td>
<td>1.5 ± 0.2</td>
<td>0.47 ± 0.05</td>
<td>0.007 ± 0.004</td>
<td>0.50</td>
<td>10.1</td>
</tr>
<tr>
<td>T. K.</td>
<td>P</td>
<td>F</td>
<td>27</td>
<td>Chest wall</td>
<td>1.38 ± 0.07</td>
<td>2.2 ± 0.5</td>
<td>0.39 ± 0.09</td>
<td>0.025 ± 0.006</td>
<td>0.65</td>
<td>12.5</td>
</tr>
<tr>
<td>T. K.</td>
<td>M1</td>
<td>F</td>
<td>27</td>
<td>Lymph node</td>
<td>1.13 ± 0.10</td>
<td>2.4 ± 1.0</td>
<td>0.47 ± 0.08</td>
<td>0.020 ± 0.009</td>
<td>0.50</td>
<td>14.7</td>
</tr>
<tr>
<td>T. K.</td>
<td>M2</td>
<td>F</td>
<td>27</td>
<td>Lymph node</td>
<td>1.47 ± 0.05</td>
<td>2.3 ± 0.4</td>
<td>0.42 ± 0.06</td>
<td>0.019 ± 0.006</td>
<td>0.61</td>
<td>11.2</td>
</tr>
</tbody>
</table>

| A. V.   | P     | M   | 22      | Forearm | 0.87 ± 0.11 | 4.6 ± 3.4 | 0.24 ± 0.10 | 0.114 ± 0.010 | 0.40 | 4.5 |
| A. V.   | M1    | M   | 22      | s.c. tissue | 0.93 ± 0.04 | 3.0 ± 0.6 | 0.45 ± 0.04 | 0.076 ± 0.006 | 0.33 | 3.8 |
| A. V.   | M2    | M   | 22      | s.c. tissue | 1.00 ± 0.06 | 2.8 ± 0.8 | 0.38 ± 0.06 | 0.077 ± 0.011 | 0.37 | 5.1 |
| A. V.   | M3    | M   | 22      | Lymph node | 0.96 ± 0.04 | 3.0 ± 0.5 | 0.46 ± 0.07 | 0.069 ± 0.012 | 0.37 | 4.4 |
| D. U.   | P     | M   | 45      | Thigh | 0.91 ± 0.05 | 1.7 ± 0.4 | 0.78 ± 0.05 | 0.041 ± 0.009 | 0.17 | 10.9 |
| D. U.   | M1    | M   | 45      | Lymph node | 0.91 ± 0.03 | 1.5 ± 0.2 | 0.91 ± 0.04 | 0.021 ± 0.007 | 0.17 | 9.0 |
| D. U.   | M2    | M   | 45      | Lymph node | 0.93 ± 0.04 | 1.7 ± 0.4 | 0.77 ± 0.05 | 0.037 ± 0.008 | 0.20 | 9.6 |
| V. B.   | P     | F   | 26      | Back | 0.84 ± 0.06 | 4.1 ± 1.6 | 0.47 ± 0.13 | 0.083 ± 0.024 | 0.41 | 6.3 |
| V. B.   | M1    | F   | 26      | s.c. tissue | 0.86 ± 0.03 | 3.3 ± 0.5 | 0.49 ± 0.05 | 0.081 ± 0.008 | 0.33 | 6.2 |
| V. B.   | M2    | F   | 26      | Lymph node | 0.85 ± 0.05 | 3.3 ± 1.0 | 0.64 ± 0.10 | 0.058 ± 0.018 | 0.36 | 5.4 |
| V. B.   | M3    | F   | 26      | Lymph node | 0.89 ± 0.07 | 3.4 ± 1.4 | 0.51 ± 0.12 | 0.070 ± 0.022 | 0.39 | 7.0 |

**Sensitive primary tumor, no heterogeneity (Group II)**

- $SF_{2}$, surviving fraction at 2.0 Gy from a single experiment on the mean from two experiments; $P_{1}$, primary tumor; $M_{1}$, metastasis no. 1; $M_{2}$, metastasis no. 2; $M_{3}$, metastasis no. 3.
- $^a$ Mean ± SE.
Group I consisted of four patients with radioresistant primary tumors; the \( D_0 \)s ranged from 1.38 ± 0.06 Gy to 1.69 ± 0.08 Gy (Fig. 1). The radiation sensitivity of the metastases was not significantly different from that of the primary tumor; i.e., no heterogeneity was observed. Group II consisted of three patients with radiosensitive primary tumors; the \( D_0 \)s ranged from 0.84 ± 0.06 Gy to 0.91 ± 0.05 Gy (Fig. 2). Heterogeneity was not observed in this group either; all metastases were as sensitive as the primary tumor. Group III consisted of three patients with a heterogeneous disease (Fig. 3). The primary tumor was radiosensitive for all patients; the \( D_0 \)s ranged from 0.85 ± 0.05 Gy to 1.03 ± 0.05 Gy. In Patient B. O., the lymph node metastasis was more resistant than the primary tumor \((P < 0.05)\), in contrast to the two s.c. metastases. Patient G. W. had developed a lymph node metastasis that was considerably more resistant than the primary tumor \((P < 0.05)\). Both metastases in Patient K. A. were also more resistant than the primary tumor \((P < 0.05)\). Moreover, the lung metastasis was more resistant than the lymph node metastasis \((P < 0.05)\) in this patient. Analyses based on the linear-quadratic model revealed that the heterogeneity of Patient G. W. could be ascribed mainly to differences in the \( \beta \) coefficient, whereas the heterogeneity of the Patient K. A. was mainly a consequence of differences in the \( \alpha \) (Table 1).

One point to note is that heterogeneity was never observed in a patient with a radiosensitive primary tumor. Furthermore, no metastasis was found to be significantly more radiosensitive than the primary tumor in any of the ten patients.

The radiation sensitivity of the tumors, whether they were primary lesions or metastatic deposits, did not seem to depend on the patient's age and sex or the tumor location. Moreover, none of the survival curve parameters listed in Table 1 showed a significant correlation to PE, as verified by linear regression analysis including data from all 30 tumors. However, there seemed to be a relationship between \( D_0 \) and PE for lesions within individual patients. Thus, the metastases showed approximately the same PE as the primary tumor in most patients where heterogeneity was not observed (Table 1, Groups I and II). If heterogeneity was observed, however, the metastases that showed a higher \( D_0 \) than the primary tumor also showed a higher PE (Table 1, Group III).

**DISCUSSION**

Development of Cellular Radiation Resistance. Tumor progression is a process which has been subjected to extensive experimental and theoretical studies since Foulds (27, 28) introduced the concept and suggested that the process involves a series of changes occurring individually in each patient. It has been hypothesized that tumor progression is a consequence of genomic instability and leads to the development of new and more malignant cell subpopulations (29, 30). The present study confirmed previous observations that human tumors can show clonal heterogeneity in phenotypic characteristics (1, 3); the cellular radiosensitivity was found to differ significantly between the primary tumor and the metastatic lesions in some patients.

Two important observations are reported here: metastases of patients with radiosensitive primary tumors showed cell survival curves similar to those of the primary tumor (Fig. 1); and metastases of patients with radioresistant primary tumors were either of the same radiation sensitivity as the primary tumor (Fig. 2) or more resistant (Fig. 3). These observations elucidate the association between the development of radiosensitive phenotypes and the development of metastatic phenotypes during tumor progression. (a) A tumor cell can become metastatic without a concomitant change in radiation sensitivity, since
both the primary tumor and the metastatic lesions were radiosensitive in some patients. (b) Some genetic alterations transforming a tumor cell from nonmetastatic to metastatic may possibly also cause radiation resistance, since some patients with radiosensitive primary tumors possessed radioresistant metastatic deposits. Observations suggesting the existence of genetic alterations causing a tumor cell to become both metastatic and radiosensitive were not made.

Hill (9) has presented evidence that tumor progression to increased levels of malignancy involves the development of metastatic potential and an increased ability to become resistant to treatment with chemotherapeutic agents. The study reported here suggests that tumor progression also includes an increased ability of the tumor cells to become radioresistant. It is therefore possible that some similar underlying mechanisms may be involved in the development of metastatic, drug-resistant, and radioresistant tumor cell phenotypes.

Radiation Treatment of Metastases. The data in Figs. 1 to 3 have also significant implications for the treatment of malignant melanoma with ionizing radiation. Disseminated malignant melanoma is usually treated with chemotherapy, but the response rate is low and the prognosis poor (31). New treatment strategies are therefore under current investigation, e.g., radioimmunotherapy (32, 33). Since the metastatic lesions can be significantly more radioresistant than the primary tumor, the therapeutic benefit of using monoclonal antibodies labeled with isotopes such as $^{131}$I and $^{90}$Y may be limited, due to the low linear energy transfer radiation and the low dose rates that are achieved. The use of $^{211}$At-labeled antibodies, which have a half-life of 7.2 h and emit $\alpha$-particles of 5.9 and 7.5 MeV, would probably be preferable from a radiobiological point of view (34). Boron neutron-capture therapy, another therapeutic approach making use of high-energy $\alpha$-particles, may also be of interest in malignant melanoma, for example, in the treatment of metastases in the brain (35).

Recent clinical investigations have indicated that the radiosensitivity of malignant melanoma differs considerably among patients; some primary tumors are resistant, whereas others respond well to external radiation treatment (36, 37). Radiobiological studies of cells isolated directly from surgical specimens have given results in agreement with these clinical observations (21). The work reported here also supports this view. It has been suggested that the treatment of malignant melanoma should be individualized on the basis of the biological properties of each patient’s tumor (21, 38, 39). Several possible predictive assays for tumor treatment sensitivity have been suggested and are under development (40–42). However, the present observation that malignant melanoma can show intrapatient heterogeneity in radiation sensitivity due to the induction of resistant cell phenotypes during tumor progression indicates that the development of a clinically useful predictive assay for this disease may be a comprehensive and difficult task.

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REFERENCES

17. Rofstad, E. K., Zaffaroni, N., and Hystad, M. E. Heterogeneous radiation and heat sensitivity in vitro of human melanoma xenograft lines established from different lesions in the same patient. Comparisons with the radiation...
RADIATION SENSITIVITY OF MELANOMA


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