Relationship of Spontaneous Regional Lymph Node Metastases to Dose of Local Irradiation of Primary B16 Melanomas

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ABSTRACT

We evaluated the effects of local X-irradiation on microscopic or small macroscopic primary melanomas in the feet of C57BL/6 mice and the subsequent development of spontaneous femoral lymph node (LN) metastases. Doses of 30, 40, 55, 62.5, or 72.5 Gy often cured the foot tumor and metastases to regional femoral lymph nodes were relatively uncommon. Doses of 3.75, 7.5, 10, 15, and 20 Gy were associated with a dose-dependent regrowth delay of the foot tumor treated at microscopic size. Foot melanomas that were not cured spread to regional femoral LNs more frequently (P < 0.001). The relative risk of developing femoral LN metastasis increased 2.55 times for each 1-mm increase in the anteroposterior diameter of the foot tumor in mice with 20 days of primary tumor exposure and increased 4.87 times for each 1-mm increase in mice with 100 days of primary tumor exposure. Although tumors treated with subcurative doses of irradiation had a longer period of time to metastasize to regional LNs for each 1-mm increase in primary tumor size, this variable alone did not account for the increased incidence of metastasis seen with irradiation.

INTRODUCTION

Spontaneous metastasis (1) to RLNs (2) is a relatively common event in the growth and spread of primary epithelial malignancies (2-4). Involvement of the RLNs by metastatic tumor is important in the survival of the animal because hematogenous metastasis and death commonly follow (2, 3, 5, 6). Factors that alter the incidence of metastasis to RLNs (2, 7-14) influence the incidence of hematogenous metastasis and survival.

The effect of irradiation on the subsequent development of hematogenous metastasis has been studied extensively in animals and humans. Increased hematogenous metastasis following irradiation of breast adenocarcinoma in women was first reported in 1919 (quoted in Ref. 15). Subsequent clinical studies confirmed this observation (15, 16). Experiments in animals also showed increased hematogenous metastases following local irradiation (17-27). There are, however, a few reports in animals and humans that fail to confirm these findings (28-31). In all of these studies the source, dose, and timing of irradiation, the species of the tumor host, and the site and histology of the local tumor differed. Perhaps even more importantly, all the tumors were treated when they were clinically measurable. The incidence of metastasis increases with increasing tumor size (3, 4, 32), and it is not possible to exclude size as a major determinant of metastasis in these previous studies.

In order to study the effects of X-irradiation on subsequent metastasis to lymph nodes, we established a model of melanoma syngeneic in C57BL/6 mice in which the size of the primary tumor in the footpad is the most important factor in determining RLN spread (3, 4). The tumors were irradiated in vivo at microscopic size such that any change in the incidence of subsequent regional lymph node metastasis would be more likely related to irradiation. In some additional experiments tumors were irradiated at 1 or 2 mm diameter when distant metastases may have been present in very low numbers. Varying doses of X-irradiation were delivered to test the hypothesis that dose would affect the incidence of subsequent regional lymph node metastasis. This paper summarizes a series of irradiation experiments in the melanoma model performed showing a dose-related increase in RLN metastases.

MATERIALS AND METHODS

Experimental Animals. Six- to eight-week-old inbred C57BL/6 mice were obtained from the National Cancer Institute, Frederick Cancer Research Facility, MD, and handled as previously described (3, 4). The mice were free of viruses, parasites, and mycoplasma. The cages were kept at constant temperature. All experiments had prior approval of the institutional animal care committee.

Tumors. The F10 variant of the B16 melanoma cell line was obtained from Drs. Fidler and Hart at the Frederick Cancer Research Facility in 1982. Cells were grown in tissue culture in Eagle's minimum essential medium with 10% fetal calf serum, nonessential amino acids, sodium pyruvate, L-glutamine, vitamin solution, penicillin, and streptomycin (M. A. Bioproducts, MD), as described previously (3, 4). Cells were harvested by mild trypsinization (0.05% trypsin, 0.02% EDTA solution, Gibco, NY). An inventory of cells was established by freezing the harvested cells in 10% dimethyl sulfoxide (Sigma, St. Louis, MO) and 10% fetal calf serum (Gibco, NY) in a programmed liquid nitrogen freezer (Cryomed, MI). Cells were stored in the vapor phase of liquid nitrogen (~180°C). Prior to tumor inoculation the cells were thawed, washed, and suspended in RPMI 1640 (Gibco, NY) without additives.

Tumor cell viability was assessed by trypan blue exclusion. The cells were maintained at a concentration of 1 x 10^6 viable cells per ml at 4°C to limit clumping prior to inoculation.

Tumor Inoculation. The mice were anesthetized by inhalation of methoxyflurane (Pittman Moore, NJ). The left footpad of each animal was injected s.c. with 5 x 10^6 viable tumor cells.

Tumor Growth. Animals were inspected daily for general health and for growth of the tumor in the left footpad. The anteroposterior diameter of the foot containing the tumor and the normal right foot were measured with standard calipers. The size of the tumor was estimated by subtracting the size of the normal right foot from the combined size of the left foot, the tumor, and irradiation-induced swelling where applicable.

X-Irradiation. Mice were randomly allocated to control or to receive a single dose of radiation. Radiation was delivered by means of a Keeler X-ray unit operating at 250 kVp, 15 mA 1.3 copper half value layer, and a target-to-source distance of 30 cm. Each mouse was restrained in a specially constructed lead shield, used to protect regional lymph nodes and the entire trunk and head, with only the foot protruding. Care was taken to immobilize the foot without obstructing the blood supply. The dosimetry was verified with a condenser chamber and meter. The dose rate was approximately 2.75 Gy per minute. All mice were similarly immobilized to control for the stress factor. Control mice received no irradiation to their tumor-bearing foot. All experiments included six to 12 mice per dose point and were reproduced at least once. The doses administered were 3.75, 7.5, 10, 15, 20, 30, 40, 55, 62.5, and 7.27 Gy. Tumors were irradiated at microscopic size 7 days after inoculation in the footpad in some experiments. In other experiments animals were irradiated when the anteroposterior diameter of the foot tumor was 1 or 2 mm.
was 1 or 2 mm. Additional groups of six mice per group had their feet irradiated without prior inoculation of tumor to assess swelling of normal tissue.

Amputation and Lymph Node Sampling. The tumor-bearing limb was amputated through the hip joint under methoxyflurane inhalation anesthesia. For some experiments this procedure was done at 1, 2, 3, 4, or 5 mm primary tumor size as measured by calipers. In some experiments 1 and 4 mm sizes were eliminated. In some experiments amputations in treated mice were performed when tumors in the respective control animals measured 1, 2, 3, 4, or 5 mm. This was done to test the hypothesis that metastasis may occur because of increased length of time irradiated tumors are present in the animal (see "Statistical Analysis"). The femoral lymph nodes were separately dissected and placed in formalin for histological evaluation. The gross appearance of the lymph node was carefully noted, especially for size and the appearance of black pigmentation. Lymph nodes were scored positive irrespective of the quantity of tumor present.

Histopathology. The resected femoral nodes were embedded in paraffin, thin sections cut, stained with hemotoxylin & eosin and evaluated histologically. Because of our previous experience in detecting melanoma metastasis in lymph nodes we only evaluated grossly negative lymph nodes histopathologically to confirm their negativity. Previously unrecognized microscopic metastases were scored positive.

Statistical Analysis. All of the 909 mice in these experiments were analyzed using a stepwise logistic regression analysis (33, 34) to test for the presence of statistically significant predictors of femoral node metastasis. Logistic regression was chosen rather than Cox proportional hazards regression analysis, as time to event (metastasis) is not observed. Animals were sacrificed at prescribed times and the presence of metastasis was ascertained at that time. However, the length of tumor exposure time (time to amputation or unplanned death) was included as an independent variable in the logistic regression to determine whether metastasis is a function of exposure time, independent from the influence of tumor size or the effect of irradiation. In addition to tumor exposure time, radiation dose level (in Gy), final tumor size (mm), and timing of irradiation (microscopic, 1 or 2 mm tumor size) were used as independent variables. Three interactions (second-order products) were also included to give a total of seven variables analyzed. The interactions included: dosage level x final tumor size, dosage level x tumor exposure time, and final tumor size x tumor exposure time. The risks of developing femoral lymph node metastasis associated with each of the selected independent variables were estimated from the final logistic regression model.

RESULTS

Effects of Irradiation Dose on Microscopic Primary Tumor Growth in the Foot. Black tumor became macroscopically visible in the second week following inoculation in mice whose tumors did not receive irradiation. The growth curves of tumors irradiated with doses 0 through 30 Gy are depicted in Fig. 1. Only occasional growth occurred in the foot after irradiation of microscopic tumors treated with irradiation doses of 40 Gy or higher.

Fig. 2 shows the percentage of mice without foot tumor at 150 days after tumor inoculation as a function of the radiation dose to the initial microscopic tumor. Twenty % of mice developed progressively growing tumors within 150 days after a tumor dose of 30 Gy and 62.5% within 9 months. Eighty-five % of mice developed progressively growing tumors after 20 Gy within 150 days after tumor inoculation. Eighty-one % of mice developed progressively growing tumors after 15 Gy within 150 days after tumor inoculation. At doses of x-irradiation below 10 Gy all mice developed progressively growing tumors within 150 days after tumor inoculation. One hundred % of mice developed progressively growing primary tumors in the foot after inoculation of $5 \times 10^4$ viable tumor cells when no irradiation is given.

Single doses of 62.5 and 72.5 Gy resulted in no tumor growth in the primary area in the foot (data not shown). Single doses of 40 Gy cured 7/8 microscopic tumors and 1/8 grew slowly to 2 mm in 5 months when leg amputation was done. Fifty-five Gy cured 7/8 microscopic tumors, and the remaining animal underwent amputation at 7 months when the tumor was 1 mm in AP diameter. Swelling of normal tissues was induced by doses of 30 Gy or higher. No discernible swelling was encountered in normal tissues following doses of 20 Gy and less. Tissue loss including ulceration and loss of toes was seen consistently after doses of 55, 62.5, and 72.5 Gy.

Effects of Irradiation Dose on Macroscopic Primary Tumor Growth in the Foot. Single doses of 7.5 Gy to tumors at 1 mm AP diameter did not affect the growth of these tumors (data not shown).

Swelling induced by irradiation doses of 30, 40, 55, 62.5, and 72.5 Gy appeared more extensive than for comparable X-ray doses delivered to feet containing no tumor or microscopic tumor but this difference was difficult to quantify exactly. All mice receiving no irradiation had their tumor-bearing limb amputated when the tumor was 5 mm in AP diameter. Mice whose tumors received 30 Gy had some swelling of normal tissues and minimal slowing of tumor growth and had amputation at 5 mm AP diameter of tumor. Animals receiving doses of 40, 55, 62.5, and 72.5 Gy suffered extensive skin damage, swelling, ulceration and clubbing of the toes and underwent amputation at 3-mm tumor size dictated by humane rules of the Animal Care Committee. In these groups of mice there was some slowing of tumor growth.

Effects of Irradiation Dose on Subsequent Development of Femoral Lymph Node Metastasis. One-hundred thirty-seven of the total 909 mice studied in these experiments had tumor metastases to regional femoral lymph nodes. Table 1 gives a listing of the statistically significant predictors of femoral node
metastasis from the final logistic regression model.

Within the logistic regression model irradiation dosage level is a statistically significant (P < 0.001) predictor of femoral node metastasis after accounting for the other potential predictors, including length of primary tumor exposure time. Taking the mice with 60 days of tumor exposure time as an example, those exposed to a 10-Gy dosage level had 2.37 times more metastatic risk than control mice. Taking the mice with 100 days of tumor exposure time as an example, those exposed to a 10-Gy dosage level had 1.97 times more metastatic risk than control mice.

Tables 2 and 3 show the incidence of femoral lymph node metastasis in seven separate experiments at particular tumor sizes after varying doses of X-irradiation to the microscopic primary tumor. The incidence of metastasis in the mice whose tumors received 40, 55, 62.5, and 72.5 Gy is relatively low overall, but this may be related to the high cure rate for primary tumors. This suggests that local control of the primary tumor prevents the potential spread to regional lymph nodes. Paradoxically, however, there is also a relatively high incidence of metastasis at smaller sizes where the primary tumor in the foot was not cured. At dose 40 Gy the one mouse that developed a 2-mm tumor had metastases, a size which in control mice rarely results in metastasis. Similarly, the one mouse that had a small tumor grow in the foot after 55 Gy developed metastases. One mouse whose tumor received 72.5 Gy developed metastases with no primary tumor growing in the foot and when metastases are not normally seen.

Table 4 shows the femoral lymph node metastases after the primary foot tumor was irradiated with doses 30 through 72.5 Gy at 2-mm size. The incidence of metastases is high for all the mice whose tumors were irradiated, particularly those whose tumor-bearing limbs were amputated at 3-mm primary tumor size.

Table 5 shows the incidence of femoral lymph node metastases in mice whose primary foot tumors were irradiated at 7.5 Gy when they had reached 1 mm in size and were macroscopically visible. In this experiment there is also an apparent increase in lymph node metastases in irradiated mice.

**DISCUSSION**

The incidence of RLN metastasis to femoral nodes was directly related to the size of the primary tumor, confirming our previous studies (3, 4). Irradiation that cured the foot tumor most often prevented metastasis to RLNs. Irradiation was purposely administered at microscopic or small macroscopic tumor sizes when minimal numbers of metastases were present in order to address this question. In contrast, in the more

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### Table 1

<table>
<thead>
<tr>
<th>Independent variable</th>
<th>Regression coefficient</th>
<th>Coefficient standard error</th>
<th>$x^2$ statistic</th>
<th>Test $P$ value</th>
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<tbody>
<tr>
<td>Size</td>
<td>0.7377</td>
<td>0.1423</td>
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<td>0.0146</td>
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<td>0.0001</td>
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<td>Size x time</td>
<td>0.0081</td>
<td>0.0023</td>
<td>12.85</td>
<td>0.0003</td>
</tr>
<tr>
<td>Dosage x time</td>
<td>-0.0005*</td>
<td>0.0002</td>
<td>4.12</td>
<td>0.0424</td>
</tr>
</tbody>
</table>

* The animals given a very high dose were often cured of the primary tumor, resulting in the absence of metastasis. In addition, the cured animals were usually given long exposure times, since they have reached the tumor sizes specified for amputation. As a consequence, the logistic analysis results indicated that the animals with a combination of higher dosages and longer exposure times had a lower risk of metastasis (a negative regression coefficient).

### Table 2

<table>
<thead>
<tr>
<th>Tumor size (mm) at amputation</th>
<th>Tumor irradiated</th>
<th>Femoral lymph node metastases after local tumor irradiation with</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3.75 GY</td>
<td>7.5 GY</td>
</tr>
<tr>
<td>1 No</td>
<td>0/7 (0)</td>
<td>0/10 (0)</td>
</tr>
<tr>
<td>1 Yes</td>
<td>0/10 (0)</td>
<td>0/10 (0)</td>
</tr>
<tr>
<td>2 No</td>
<td>0/10 (0)</td>
<td>0/10 (0)</td>
</tr>
<tr>
<td>2 Yes</td>
<td>0/10 (0)</td>
<td>0/10 (0)</td>
</tr>
<tr>
<td>3 No</td>
<td>0/10 (0)</td>
<td>0/10 (0)</td>
</tr>
<tr>
<td>3 Yes</td>
<td>0/10 (0)</td>
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<td>5 No</td>
<td>0/10 (0)</td>
<td>0/10 (0)</td>
</tr>
<tr>
<td>5 Yes</td>
<td>0/10 (0)</td>
<td>0/10 (0)</td>
</tr>
</tbody>
</table>

* Number of mice with metastases over total number of mice. Numbers in parentheses represent % of mice with metastases.

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time and this may account for the negative regression coefficient in Table 1. This growth delay results in the mice being exposed to tumor for a longer time. Although it is possible that such irradiated tumor cells have potential access to lymphatics for a longer time, our experiments show that this factor alone does not account for increased metastasis.

Metastasis is a complex process involving a number of steps (36). Tumor cells with poor metastatic potential can be induced to increase their metastatic capability by changing their genetic makeup (37) by transfection of oncogenes. Drugs have been used to promote metastasis by activation of previously quiescent genes (38). Sublethal irradiation induces chromosomal changes in tumor cells (1) and could possibly activate genes, resulting in functional changes in the cell responsible for metastasis.

Irradiation could change metastatic potential by indirect mechanisms. Preliminary WBI of rodents enhances the incidence and numbers of metastases from transplanted tumor (27). Immune response of the host to immunogenic and nonimmunogenic (39) tumors are inhibited by WBI. Our results are unlikely to be due to accidental WBI because each mouse was carefully shielded with a lead screen. Because of the small size of the mouse hindfoot, the possibility of irradiation scatter, and the mobility of mice in their restraining device, it is possible that RLN in some mice may have received some irradiation. However, it is unlikely that this could account for the irradiation-induced enhancement of metastasis to femoral nodes. Hewitt et al. (39) could induce increased metastasis of a poorly immunogenic mammary carcinoma to the lungs of mice whose lungs were previously irradiated. In this same model prior irradiation of lymph nodes did not increase the incidence of RLN metastasis after intradermal tumor implantation. Fisher (27) reported metastases to lungs of Sprague-Dawley rats and syngeneic C3H mice after curative irradiation of Walker or mammary carcinomas inoculated into the legs. In these experiments metastases in the lymph nodes were decreased after irradiation with 5000 rads. In accompanying experiments he showed increased metastases in lungs or liver after irradiation of these organs prior to the tumor inoculation.

Irradiation of the host tissue prior to inoculation of syngeneic tumor in experimental systems may inhibit the growth rate of established tumors (25, 26). This TBE may be associated with increased lung metastases (26). Although the classic TBE requires prior exposure of the normal tissue to irradiation, it is possible that local host tissue changes induced by irradiation of the tumor could account for the increased femoral node metastases. Endothelial cells of blood vessels are important in the metastatic cascade (36). Tumor cells enter the circulation by invasion of the capillary endothelium. These neoplastic cells extravasate and form metastases by adhering to capillary endothelium and extravasating through the basement membrane. Endothelial cells of lymphatics probably interact with cancer cells in similar ways, although this has not been studied extensively in the lymphatics related to the primary tumor (40). Irradiation-induced vascular changes may enhance the ability of tumor cells to invade capillaries (40, 41). We cannot exclude this phenomenon as a possible explanation for our results although Milas (42) has shown that TBE does not occur in tumors less than 5 mm in diameter.

Thorkild Roving, at a meeting of the Society of Danish Surgeons in 1919, described increased hematogenous metastases after Roentgen irradiation of malignant breast tumors [reported by Krebs (15)]. Because of the techniques of the irradiation and the relatively poor knowledge of modern clinical investigation used in this and other studies cited by Krebs from the early part of the century (quoted in Ref. 15), such conclu-

### Table 3 Femoral lymph node metastases after irradiation doses 30 through 72.5 Gy given at microscopic tumor size

<table>
<thead>
<tr>
<th>Irradiation dose</th>
<th>Size at amputation (mm)</th>
<th>Mice with metastases, total N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5</td>
<td>2/8 (25)</td>
</tr>
<tr>
<td>30</td>
<td>0</td>
<td>0/2 (0)</td>
</tr>
<tr>
<td>30</td>
<td>3</td>
<td>0/1 (0)</td>
</tr>
<tr>
<td>40</td>
<td>4</td>
<td>2/2 (100)</td>
</tr>
<tr>
<td>40</td>
<td>5</td>
<td>3/3 (100)</td>
</tr>
<tr>
<td>55</td>
<td>0</td>
<td>0/7 (0)</td>
</tr>
<tr>
<td>55</td>
<td>1</td>
<td>1/1 (100)</td>
</tr>
<tr>
<td>62.5</td>
<td>0</td>
<td>0/8 (0)</td>
</tr>
<tr>
<td>72.5</td>
<td>0</td>
<td>1/8 (13)</td>
</tr>
</tbody>
</table>

Table 3 typically shows the incidence of metastases after varied doses of irradiation on tumors of different sizes. The data in Table 1 indicates that the growth of the primary tumor is delayed. Exposure time to primary foot tumor is cured. The phenomenon of RLN metastasis occurs in all dose ranges of X-irradiation where the growth rate of the primary tumor is delayed. Exposure time and growth to final tumor size are interdependent variables. Irradiation delays growth rate and results in increased exposure time and this may account for the negative regression coefficient in Table 1. This growth delay results in the mice being exposed to tumor for a longer time. Although it is possible that such irradiated tumor cells have potential access to lymphatics for a longer time, our experiments show that this factor alone does not account for increased metastasis.

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