Tumor Induction in Mice Locally Irradiated with Carbon Ions: A Retrospective Analysis

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Leg/LET/Fractionation/Dose/Histology.

Tumor induction in mice legs that were locally irradiated with carbon ions was compared to tumor induction by γ rays after single and fractionated irradiation. A total of 250 tumors were induced in 1104 mice that received carbon-ion doses of 5 through 65 Gy. A total of 77 tumors were induced in 371 mice that received γ-ray doses of 45 through 95 Gy. Of 91 carbon-ion induced tumors examined histologically, 97 percent were malignant, and sarcomas such as malignant fibrous histiocytoma (47%) and fibrosarcoma (32%) were most frequently observed. Malignant fibrous histiocytoma was also the most frequently observed tumor (12 out of 20 tumors; 60%) after γ-ray irradiation, followed by carcinomas (25%) such as adenocarcinoma and squamous cell carcinoma. Neither dose fractionation nor linear energy transfer affected tumor induction for carbon ions and γ rays. Dose responses were linear for carbon ions and γ rays, and showed no saturation up to 65 Gy of carbon ions and 95 Gy of γ rays. The relative biological effectiveness of carbon ions was 2.2 for tumor induction and 1.9 for early skin reaction. We conclude that risk of secondary tumor induction by carbon-ion radiotherapy would not be seriously higher than anticipated.

INTRODUCTION

Heavy-ion radiotherapy at the National Institute of Radiological Sciences (NIRS) treated more than 1,000 patients by Year 2001 ([http://www.nirs.go.jp/report/hene/h13/04/57.htm]), and has treated more than 1,700 patients by Year 2004. Carbon ions, high LET (linear energy transfer) radiation, are strong in its biological activities such that biological effectiveness relative to photons (RBE) of 2.4 is used for beam’s spread-out Bragg peak (SOBP) that consists of the high LET radiation.¹ LET of carbon-ion beams are heterogeneous within irradiated body in such that the therapeutic carbon beams are designed to deposit relatively low LET radiation at body entrance plateau but high LET SOBP at deeply seated tumors. We have investigated the dependence of the RBE on the carbon ion LET for in vitro cell lethality²,³,⁴) and in vivo skin reaction.⁵) These biological data have been used to design the ridge filters that provide a uniform distribution of biological effectiveness within the SOBP.¹) In the clinic, fractionated radiotherapy has been widely used. As the RBE depends not only on the LET but also on the fraction size or the dose per fraction, we have also studied the RBE for the fractionated irradiation of the various LET carbon beams using mouse legs skin reaction.⁶) When we observed legs further time after irradiation, some mice developed tumors at the irradiated legs. We here retrospectively analyzed and reported tumor induction in the irradiated legs after carbon-ion irradiation, and compared with tumor induction after γ-ray irradiation. RBE of carbon ions for tumor induction was also compared with RBE for early skin reaction.

MATERIALS AND METHODS

Mice and irradiation

C3H/HeMsNrfs female and male mice that aged 12–18 week-old were used for this study. The mice were produced and maintained in the specific pathogen-free (SPF) facilities in our institute. Hairs on the mouse right hind leg were removed by applying a depilatory agent before the first irradiation. A total of 1475 mice were used in these experiments with 5 mice for each irradiation scheme. Carbon-12 ions were accelerated by either the HIMAC synchrotron up to 290 MeV/u or the RIKEN Ring cyclotron up to 135 MeV/u. Exposures were conducted using horizontal carbon beams with a dose rate of approximately 3 Gy/min. The LET of 290 MeV/u carbon ions was 14 keV/μm.

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at the entrance of a 6-cm SOBP (Spread-Out-Bragg-Peak), and gradually increased to 20 keV/μm along the 8-cm beam path (1 cm before the proximal edge of the SOBP). The proximal edge of the SOBP was located at 9-cm path from the entrance, and the LET at this position was 40 keV/μm. The LET further increased with beam path and reached 200 keV/μm or greater at 15-cm path, i.e., at the distal fall-off of the SOBP. The irradiation fields were defined by use of an iron and a brass collimator. The depth position along irradiation path was adjusted by a Lucite range shifter. The LET of 135 MeV/u carbon ions was 22 keV/μm at the entrance of monoenergy beam, and gradually increased up to 300 keV/μm or greater at the unmodulated Bragg peak.

With pentobarbital anesthesia (50 mg/Kg) and taping, five mice were immobilized on a Lucite plate to place right hind legs in a rectangular field of dimensions 28 × 100 mm, and received either a single dose or fractionated irradiation. The foot was excluded from the irradiation field.

The reference beam consisted of Cs-137 γ-rays with a dose rate of 1.6 Gy/min at an FSD of 21 cm. A doughnut-shaped radiation field with 30-mm rim was used to collimate the vertical beam.

Fractionation varied in both time intervals and number of fractions. The most frequent time interval was 24 hr while some fractionation was made within as short as 2 hr. Number of fractions ranged from one to 16.

Skin reaction
Skin reactions of the irradiated legs were scored every other day, starting from Day 7 after initial irradiation up to the Day 35. Our scoring scale consisted of 10 steps, ranging from 0.5 to 5.0. The five highest scores in an individual mouse were averaged, and this averaged score was designated the skin reaction score.

Data acquisition and analysis
Irradiated legs of mice were palpated once a month for up to 400 days with some exception where observation period was extended to 800 days. Number of tumors appeared at an irradiated leg was one in any mice developed tumors. We measured by calipers three diameters of a tumor at initial observation, and counted as one when the tumor grew at next measurements. Tumors appeared in unirradiated site was excluded from analysis.

RESULTS
Time of tumor appearance
The initial day of fractionated irradiation was defined as Day 0. A total number of mice irradiated with carbon ions...
was 1104. Of 250 induced tumors, the earliest tumor appeared after the carbon-ion irradiation was observed at Day 141, while the latest tumor was detected at Day 616 (Fig. 1). For γ-ray irradiation, 371 mice were used and 77 mice developed tumors. The earliest tumor appeared at Day 230 while the latest one was at Day 774. We did not find any difference between carbon ions and γ rays in the latency of tumor induction after irradiation.

**Dose versus tumor-incidence relation**

As the number of animals for each dose was too small to obtain an incidence of tumor, we grouped several doses by size ensuring the number of each group as minimum 35. The grouping forced data points to include different LET and different fractionation. Fig. 2a shows the relation between dose and tumor-incidence for carbon ions and for γ rays. The tumor incidence was 10% at a carbon dose of 5 Gy (median value), and linearly increased with dose up to 65 Gy. The linear increase was also observed for γ rays. When extrapolated to zero Gy, the regression lines of carbon-ion groups and of γ-ray groups hit to ~ 8%. Biological effectiveness of carbon ions relative to γ rays was 2.2 when the tumor incidence of 20% was used as an isoeffect. The reason we used 20% as isoeffect was that this value was the middle point on the dose response curves for both carbon ions and γ rays. Dependence on LET was also noticed in such that 60 keV/μm carbon-ions showed a steeper dose-incidence relation than 20 and 100 keV/μm carbon-ions (Fig. 2b). It should be noted that penetration depth of 100 keV/μm carbon ions was so shallow that the beam entrance side of legs were irradiated while the beam exit side of legs did not receive radiation.

**LET versus tumor-incidence relation**

Tumor incidence was analyzed for LET dependence. Here we again grouped several LET by size ensuring the number...
of each group as minimum 35. Fig. 3a shows the relation between LET and tumor-incidence for carbon ions and γ rays. Tumor incidence was 21% for γ rays. Carbon-ions of 42 and 75 keV/μm induced tumors in 18% of irradiated mice whereas those of 50 keV/μm induced tumors in 29% of irradiated mice. No significant difference was detected between γ rays and carbon ions. However, subdividing the LET groups by dose size shows again that tumor incidence increased when dose increased from 13 Gy to 35 Gy (Fig. 3b).

**Fractionation versus tumor-incidence relation**

Tumor incidence was analyzed for fractionation dependence. For either carbon ions or γ rays, no dependence of tumor induction on fractionation was detectable from 1 to 8 fractions (Fig. 4). Lowest incidence was 13% for 7 fractions with carbon ions whereas highest incidence was 28% for 4 fractions of carbon ions. Gamma rays induced 18%–22% tumors in irradiated mice (Fig. 4).

**Early skin reaction**

Early skin reaction in mice was observed between 7 and 35 days after irradiation for both carbon ions and γ rays. We retrospectively selected mice that later developed tumors, and analyzed skin reaction score for dose dependence. Here again, we grouped several doses by size ensuring the number of each group as minimum 5. As shown in Fig. 5, skin reaction increased with dose for carbon ions and γ rays as well. Carbon ions showed higher skin reaction than γ rays, and showed a left-shifted dose response. When we used skin reaction score of 3.0 as an isoeffect, biological effectiveness of carbon ions was 1.9 (56 Gy /30 Gy).

**Histology**

For histological analysis, 111 tumors that appeared early after irradiation were used (Table 1). Of 91 carbon-ion induced tumors histologically examined, 88 (97%) was malignant, and sarcomas such as malignant fibrous histiocytoma (43 tumors; 47%) and fibrosarcoma (29 tumors; 32%) were most frequently observed. One tumor was epitheloid sarcoma, possessing epithelial tissue-like appearance. Malignant fibrous histiocytoma was also the tumor most frequently observed after γ-ray irradiation (12 out of 20 tumors; 60%), followed by carcinomas (25%) such as adenocarcinoma (3 tumors) and squamous cell carcinoma (2 tumors). Three fibrosarcomas out of 20 malignant tumors (15%) were also induced by γ-ray irradiation. It is noted that all 6 osteosarcoma were induced by 135 MeV/u carbon ions without beam-spread modulation.

**DISCUSSION**

Sarcomas such as malignant fibrous histiocytomas and fibrosarcomas were predominantly induced by radiation in the present study. This contrasts with a report using rats that develop mainly carcinomas but few sarcomas. Carbon ions seem to induce sarcomas more frequently than γ rays; all osteosarcomas were induced by carbon ions but not by γ rays. Osteosarcomas are well known tumors induced by plutonium, a bone-seeking radioisotope with high LET charac-

### Table 1. Histology of induced tumors.

<table>
<thead>
<tr>
<th>Pathological diagnosis</th>
<th>Number of tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Carbon ions</td>
</tr>
<tr>
<td>Benign tumors</td>
<td></td>
</tr>
<tr>
<td>Fibroma</td>
<td>1</td>
</tr>
<tr>
<td>Osteoma</td>
<td>2</td>
</tr>
<tr>
<td>Malignant tumors</td>
<td></td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>4</td>
</tr>
<tr>
<td>Epithelioid sarcoma</td>
<td>1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lymphoma</td>
<td>1</td>
</tr>
<tr>
<td>Fibrosarcoma</td>
<td>29</td>
</tr>
<tr>
<td>Malignant fibrous histiocytoma (MFH)</td>
<td>43</td>
</tr>
<tr>
<td>Osteosarcoma</td>
<td>6&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total</td>
<td>91</td>
</tr>
</tbody>
</table>

<sup>a</sup>: 2 tumors were mixed with either adenoma or squamous cell carcinoma

<sup>b</sup>: one tumor was mixed with MFH

<sup>c</sup>: sarcoma possessing epithelial tissue-like appearance

<sup>d</sup>: all tumors were induced by monopeak of carbon ions
ter. It is possible that not only local deposit but also high LET is critical to osteosarcomagenesis. Malignant fibrous histiocytomas and fibrosarcomas are frequent as secondary tumors induced by photon radiotherapy, and are also frequent as human spontaneous tumors as well. As the cellular origin and molecular characteristics of malignant fibrous histiocytoma are poorly understood, local radiation would be useful for further providing a new animal model for sarcomagenesis.

Prominent dose dependence was observed in the present study. Incidence of tumor induction increased linearly with an increase of dose, and was not saturated nor declined at doses as large as 95 Gy of γ rays and 65 Gy of carbon ions (Fig. 2a). This contrasts with other radiation-induced tumors that show a bell-shaped dose response. It is generally accepted that decline of incidence after large dose is due to reduction of target cells surviving radiation damages. If this holds in the present results, the linear, non-saturated dose response would be due to either (1) target cells are radioresistant, (2) the population size of target cells are fairly large, or (3) target cells are those received lower than the prescribed dose at penumbra near collimators. Alternative explanation for the linear, unsaturated increase is the repair of sublethal damages. In fact, fractionated irradiation with electrons to rats increases skin tumor induction. When the regression lines of carbon ions and γ rays in Fig. 2 are extrapolated to zero dose, both lines hit 8%, which is higher than zero. It is probable that dose response curves at low doses would be steeper than those at high doses.

Dependence on LET was less prominent in the present study. LET dependence was observed only when data points were subdivided according to dose (Fig. 3b). This means that dose is the strongest determinant in tumor induction while LET and fractionation are easily influenced by the low resolution of data collection that is inevitably associated with grouping.

Tumor induction least depended on fractionation. Rat skin tumors decrease when 10 Gy of accelerated electrons is split to two equal exposures separated by 6 hr, while a total of 23 Gy rather increases skin tumors. A similar increase is also reported for lung tumor induction after fractionated X-ray radiation to C3H mice, the same strain of mice used in the present study.

RBE of carbon ions for tumor induction in the present study was 2.2. This value is similar to what is reported for the induction of mouse Hardrian gland tumors after 670 MeV/u neon ions with 25 keV/μm. Small RBE values are also known for rat skin cancer such that an RBE value 2.5 is reported for large doses of 125 keV/μm argon ions. No report has been found for RBE values of carbon ions in tumor induction. As RBE of 2.2 for tumor induction is close to 1.9, i.e., RBE for early skin reaction, risk associated with carbon-ion therapy would not be seriously higher than anticipated.

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