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BIOLOGY CONTRIBUTION

NEOPLASTIC TRANSFORMATION INDUCED BY CARBON IONS

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Purpose: The objective of this experiment was to compare the oncogenic potential of carbon ion beams and conventional photon beams for use in radiotherapy.
Methods and Materials: The HeLa X human skin fibroblast cell line CGL1 was irradiated with carbon ions of three different energies (270, 100, and 11.4 MeV/u). Inactivation and transformation data were compared with those for 15 MeV photons.
Results: Inactivation and transformation frequencies for the 270 MeV/u carbon ions were similar to those for 15-MeV photons. The maximal relative biologic effectiveness (RBE_α) values for 100MeV/u and 11.4 MeV/u carbon ions, respectively, were as follows: inactivation, 1.6 ± 0.2 and 6.7 ± 0.7; and transformation per surviving cell, 2.5 ± 0.6 and 12 ± 3. The curve for dose-transformation per cell at risk exhibited a maximum that was shifted toward lower doses at lower energies.
Conclusions: Transformation induction per cell at risk for carbon ions in the entrance channel was comparable to that for photons, whereas for the lower energies, 100 MeV/u and 11 MeV/u, which are representative of the energies delivered to the tumor margins and volume, respectively, the probability of transformation in a single cell was greater than it was for photons. In addition, at isoeffective doses with respect to cell killing, the 11.4-MeV/u beam was more oncogenic than were photons. © 2009 Elsevier Inc.

Carbon ions, Neoplastic transformation, Clonogenic survival, Relative biologic effectiveness, CGL1 cells.

INTRODUCTION

The good ability of irradiation with carbon ions to conform to the tumor shape and achieve high local control rates with low toxicity (1, 2) makes this a particularly attractive modality for irradiation of tumors in children, in whom preventing the development of secondary tumors in the margins receiving a low dose of radiation is more important than it is in adults, primarily because of children's longer life expectancy (3–5). However, clear-cut data regarding the oncogenic potential of carbon ions are lacking.

In vitro oncogenic transformation experiments have proved to be a valuable tool for providing data with good predictive power. The C3H10T1/2 rodent cell line (6) was the first to be used in quantitative studies evaluating a wide range of radiation types (7–13). Furthermore, experimental data on neutrons from a study by Miller *et al.* (14) verified the neutron quality factors and confirmed that the transformation incidence depended on neutron energy, as defined by the International Commission on Radiation Units and Meaurements (15) and the International Commission on Radiation Protection (16).

The human hybrid (Hela X skin fibroblast) cell line CGL1 has been developed by Stanbridge *et al.* (17) and adapted by Redpath *et al.* (18) for quantitative studies of radiation-induced neoplastic transformation *in vitro*. One of the most attractive features of the CGL1 system is that tumorigenicity is associated with the expression of a cell surface protein, an intestinal alkaline phosphatase (IAP) (19). Subsequently, a staining technique was developed that uses the alkaline phosphatase chromogenic substrate Western Blue to directly detect cells expressing IAP (20). In the study we report here, we used CGL1 cells to compare the oncogenic potential of monoenergetic carbon beams in reference to 15 MeV photon beams. Carbon ion beams of different energies (*i.e.,* 270 MeV/u, 100 MeV/u, and 11.4 MeV/u) were chosen to represent a therapeutic beam at the entrance channel in normal

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tissues, in the tumor margins, and in the tumor volume, respectively. Photon beams of 15 MeV were used to simulate conventional radiotherapy. Clonogenic survival, transformation per surviving cell, and transformation per initial cell at risk (or irradiated cell) were determined in the 0.2- to 3-Gy dose range for carbon ions and the 1- to 4-Gy dose range for photons.

METHODS AND MATERIALS

Cell line

CGL1 cells (a kind gift of L. Redpath, University of California–Irvine, Irvine, CA) were used in all experiments. Details on the cell culture method have been reported previously (21). Briefly, the cells were maintained in minimal essential medium supplemented with 10% fetal calf serum, 2 mmol/l glutamine, nonessential amino acids, 50 μg/l gentamicin, and 10 mmol/l HEPES. Cells were incubated with 2% CO_2. The cells were tested to confirm that they were mycoplasma free.

Survival and oncogenic transformation assays

For the high energies (270 and 100 MeV/u carbon ion beams) and photon experiments, exponentially growing cells were seeded into T25 flasks at a density of 5×10^5 cells per flask ~18 to 20 h before irradiation. One or two flasks were irradiated for each dose point. A different exposure set-up was used for the lowest-energy irradiation (11.4 MeV/u), as follows: ~ 20 h before irradiation, the cells were centerplated (1.5-cm diameter spot) in 35-mm diameter Petri dishes at a density of ~5×10^5 cells per spot (22). One to four dishes, depending on the dose, were used for each dose point. Because of the limited particle range, the dishes were irradiated without medium or a cover. For the transformation assay, we used a protocol similar to that of Redpath *et al.* (18), Sun *et al.* (23), and the Western Blue–based staining procedure of Mendonca *et al.* (20). Briefly, after irradiation, the cells were removed by trypsinization, counted, and diluted, and then plated in T75 flasks at cell concentrations that achieved close to 50 viable cells/cm^2. After 21 days of incubation, during which the growth medium was changed once a week, the cultures were fixed with 2% paraformaldehyde/phosphate-buffered saline solution for 20 min, stained with Western Blue, and rinsed with phosphate-buffered saline. Blue-stained foci were scored against a white background.

The mean number of foci per flask was calculated from the number of flasks free of transformed colonies, based on the assumption that the number of foci per flask follows a Poisson distribution (24). This method prevents overestimation of the mean number of radiation-induced foci per flask because of the possible presence of satellite foci that have formed in flasks already containing one or more foci. Data from previous studies (21, 23) have shown that the transformation frequency for the CGL1 cell line decreases with the increasing density of viable cells, according to the following equation: $T(d_1) = (d_2/d_1)^{0.407} \times T(d_2)$, where $T(d_1)$ and $T(d_2)$ are the transformation frequencies at the viable cell density d_1 and d_2, respectively. Following the convention used in studies reported elsewhere in the literature (23), the transformation frequencies were corrected using the above equation for determining cell density dependence and assuming a density of 50 cells/cm^2 as the standard.

For each experiment, in parallel with the transformation assay, small aliquots of the cell suspension were also plated in T25 flasks for evaluation of the surviving cell fraction. The number of cells plated was such as to yield ~200 viable cells/flask. Five to 10 replicative flasks were plated for each dose. The flasks were then incubated for 12 to 13 days, after which the cells were fixed with ethanol for 10 min and stained with 10% Giemsa solution. Colonies consisting of more than 50 cells were considered survivors. The plating efficiency was ~0.50.

Irradiation

Photon irradiation was performed at the Istituto Tumori in Milano using 15 MeV photons from a linear accelerator (MEVATRON 77, Siemens X15MV, Siemens Medical Laboratories Inc., Walnut Creek, CA).

Carbon ion irradiation with 100 and 270 MeV/u was performed in the medical exposure set-up at the Gesellschaft für Scherionenforschung (GSI) heavy ion synchrotron (Schwer Ionen Synchrotron [SIS], Darmstadt, Germany); carbon ion irradiation with 11.4 MeV/u was performed in the cell biology set-up at the GSI Universal Linear Accelerator (UNILAC). Details on the beam characteristics and dosimetry have been reported elsewhere (25–27). The physical characteristics of the carbon ion beams are given in Table 1. Energies on target, linear-energy transfer (LET) values, and residual ranges were calculated with ATIMA (ATomic Interaction with MAtter), a user program developed at GSI (28). The fluence contamination with lighter fragments because of deceleration by the exit window and the ionization chamber is 2% or less. Less than 1% of the total dose consists of lighter fragments. The total thickness of the living cells measured by confocal laser microscopy is 5.7 ± 0.9 μm (29). The variation in LET within the sample is ~1% for the lowest energy and smaller for the higher energies. During irradiation at the UNILAC, particle fluence was measured by an ionization chamber calibrated in reference to CR39. At the SIS, dosimetry was performed using the standard methods also used for patient treatments (30, 31).

The doses were calculated on the basis of particle fluence and the LET values given in Table 1, according to the following equation:

$$Dose[Gy] = Fluence\left[P/cm^2\right] \times LET[keV/\mu m] \times 1.602x10^{-9} \times 1/\rho\left[cm^3/g\right]$$

The irradiation time was in the range of 1 to 3 min, depending on the dose and accelerator conditions. The dose rate was in the range of several Gray per minute, and no dose rate effects were expected in this range.

RESULTS

Clonogenic survival

Figure 1a shows the survival data for all of the beams as a function of dose. The curves are the linear quadratic fits to the experimental points. The resulting values for α and ß are given in Table 2. The following relative biological effectiveness (RBE) values are shown: RBE_α calculated from the initial slope as $RBE_\alpha = \alpha_C/\alpha_{photon}$, RBE_{55} at 55% survival that

Table 1. Physical characterization of carbon beams used

Energy (MeV/u)	Energy on target (MeV/u)	LET (keV/μm)	Range in water (mm)
270.0	266.4	13.8	137
100.0	86.5	29.5	19.8
11.4	9.65	172	0.41

Abbreviation: LET = linear-energy transfer.

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