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BIOLOGY CONTRIBUTION

DIFFERENT MECHANISMS OF CELL DEATH IN RADIOSENSITIVE AND RADIORESISTANT P53 MUTATED HEAD AND NECK SQUAMOUS CELL CARCINOMA CELL LINES EXPOSED TO CARBON IONS AND X-RAYS

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Purpose: We initiated studies on the mechanisms of cell death in head and neck squamous cell carcinoma cell lines (HNSCC) since recent clinical trials have shown that local treatment of HNSCC by carbon hadrontherapy is less efficient than it is in other radioresistant cancers.

Methods and Materials: Two p53-mutated HNSCC cell lines displaying opposite radiosensitivity were used. Different types of cell death were determined after exposure to carbon ions (33.6 and 184 keV/μm) or X-rays.

Results: Exposure to radiation with high linear energy transfer (LET) induced clonogenic cell death for SCC61 (radiosensitive) and SQ20B (radioresistant) cells, the latter systematically showing less sensitivity. Activation of an early p53-independent apoptotic process occurred in SCC61 cells after both types of irradiation, which increased with time, dose and LET. In contrast, SQ20B cells underwent G2/M arrest associated with Chk1 activation and Cdc2 phosphorylation. This inhibition was transient after X-rays, compared with a more prolonged and LET-dependent accumulation after carbon irradiation. After release, a LET-dependent increase of polyploid and multinucleated cells, both typical signs of mitotic catastrophe, was identified. However, a subpopulation of SQ20B cells was able to escape mitotic catastrophe and continue to proliferate.

Conclusions: High LET irradiation induced distinct types of cell death in HNSCC cell lines and showed an increased effectiveness compared with X-rays. However, the reproliferation of SQ20B may explain the potential locoregional recurrence observed among some HNSCC patients treated by hadrontherapy. An adjuvant treatment forcing the tumor cells to enter apoptosis may therefore be necessary to improve the outcome of radiotherapy. © 2009 Elsevier Inc.

HNSCC, Carbon ion irradiation, High and low-LET irradiation, Apoptosis, Mitotic catastrophe.

INTRODUCTION

Hadrontherapy offers several advantages over conventional radiotherapy because of the physical and biological properties of carbon particles (1). The well-defined range and small lateral beam spread enable dose delivery with millimeter precision. In addition, carbon ions have an enhanced biological effectiveness in the Bragg peak region caused by the dense ionization, resulting in reduced DNA repair (2). More effective damage to the tumor at a given physical dose without causing additional damage to healthy tissues can therefore be achieved using ion-beam radiotherapy.

Several reports have investigated the biological effectiveness of carbon ions, and the results obtained confirm that high linear energy transfer (LET) ions are more effective than X-ray irradiation in killing tumor cells (3). Nevertheless, in only a few studies the mechanisms of cell death involved

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Conflicts of interest: none.

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in response to carbon ion exposure have been examined, where most of them are focusing only on apoptosis (4, 5). Conversely, in response to X-rays, premature-senescence, necrosis, and mitotic catastrophe have been described as alternative processes of proliferation inhibition or cell death in cancer cells (6, 7). Mitotic catastrophe, also known as mitotic death, has been widely described in p53-mutated tumors that are resistant to genotoxic damage. It has been suggested that this process is initially characterized by chromosome missegregation followed by aberrant mitosis or imperfect cell division, leading to the formation of multinucleated cells. Mitotic catastrophe seems to be highly dependent on DNA-damage and the subsequent failure of cell cycle arrest in G2/M phase. Moreover, the involvement of apoptotic cell death or mitosis restitution has been suggested at the end of this process (8). The contribution of mitotic catastrophe in response to carbon ion irradiation has not yet been investigated and needs to be clarified.

To improve our understanding of the biological effectiveness of carbon ions, we aimed to characterize the various patterns of cell death in HNSCC cell lines. Up to now, carbon ions have been used to treat head and neck cancers at the Heavy Ion Medical Accelerator in Japan and at the Gesellschaft für SchwerIonenforschung (GSI) in Germany. The treatment results obtained were promising and showed a favorable local control rate in adenoid cystic carcinoma (90%) and malignant melanoma (100%) (9). However, an unexplained lower control rate of 34% at 5 years was observed in HNSCC patients who developed locoregional recurrence (10). To investigate possible biological characteristics of the particular tumors able to account for the failure of carbon ion treatment, we used two HNSCC cell lines exhibiting differing radiosensitivity in the colony-forming assay. We studied the specific cell death signature of carbon ion beams at two LET values compared with X-rays. Elucidating the molecular mechanisms involved may lead to further improvement of hadrontherapy, increasing the therapeutic gain for the treatment of radioresistant tumors.

METHODS AND MATERIALS

Cells and cell-culturing conditions

The radiosensitive SCC61 cell line used in this study was derived from the tongue of a cancer patient and the SQ20B radioresistant from the larynx of another patient. Cell lines with no more than 20 passages were cultured in 95% humidity and 5% of CO_2 as previously described (11). SCC61 and SQ20B cells have similar doubling time and plating efficiency (22 hours–21% and 24 hours–28%, respectively).

Irradiation procedures

The irradiation procedures were as previously described (12). In brief, X-ray irradiation with 6 MV was performed in Lyon-Sud (France) and with 250 kV at GSI (Germany). Irradiations with 72 MeV/u carbon ions (LET 33.6 keV/μm) were performed at GANIL (France) and with 9.8 MeV/u carbon ions (LET 184 keV/μm) at GSI.

Analysis of clonogenic cell survival

Clonogenic cell survival was monitored using two protocols depending on specific technical requirements at each irradiation facility. The first method was used for the 6 MV X-ray and 33.6 keV/μm carbon ion irradiations. Both cell lines were seeded in 25 cm^2 flasks at different cell densities depending on the dose of radiation. Cells were irradiated at doses varying from 0.5 to 6 Gy.

The second method was used for 250 kV X-ray and 184 keV/μm carbon ion exposures at doses ranging from 0.5 to 6 Gy. The cells were seeded before irradiation and reseeded immediately after exposure into flasks of 25 cm^2 at different concentrations.

Cell survival was assessed by the standard colony formation assay as described in (12).

Flow cytometry analysis

The cell cycle distribution was analyzed as described previously (11).

Terminal-transferase-dUTP-nick-end-labeling (TUNEL) reaction was carried out by labeling cells with the *in situ* Cell Death Detection Kit (Promega, Charbonniére, France) according to the manufacturer's instructions.

Beta-galactosidase assay

At the indicated times, cells were washed with phosphate buffered saline; fixed in formaldehyde and glutaraldehyde at final concentrations of 2% and 0.2%, respectively; and stained overnight in X-gal staining solution at pH 6 (6). A minimum of 500 cells were scored on three slides, and the ratio of β-gal positive cells/β-gal negative cells was established.

Cellular morphologic characteristics

Morphological characteristics were visualized by means of 4′-6′ diamidino-2-phenylindole-dihydrochloride (DAPI) staining (11). Apoptotic, giant, or multinucleated cells and anaphase bridges were scored by fluorescence microscopy. A minimum of 500 cells were counted on two or three slides per experiment.

Electron microscopy

After irradiation, cells were fixed with 2% glutaraldehyde. Cells were centrifuged at 300 *g* for 10 min at room temperature and washed in 0.1 M sodium cacodylate buffer. The pellets were coated in agarose and postfixed in 1% osmium tetroxide. After washing, samples were dehydrated by graded series of ethanol and finally embedded in Epon. Observations were realized using a Philips CM120 electron microscope.

Western blot analyses

Western blot analyses were performed as previously described (13). The monoclonal antibodies used were anti-Caspase-2, anti-Chk1-P, anti-Cdc2-P (Cell Signaling Technology, Danvers, MA), anti-TP53, anti-p21 (Transduction Laboratories, Le Pont de Claix, France), anti-α-tubulin (Sigma, St. Quentin Fallavier, France) and anti-glyceraldehyde-3-phosphate-deshydrogenase (GAPDH) (Biodesign International, Saco, ME). Densitometric analysis was performed using 1DScan-EX3.1 software.

Cellular proliferation

The proliferative capacity of cells was determined using the 5-bromo-2′-deoxy-uridine (BrdU)-labeling kit (Roche, Germany) according to the manufacturer's instructions.

RESULTS

p53 status in the HNSCC cell lines

To investigate the p53 status of both HNSCC cell lines, sequencing analysis was performed in the Dr. Pierre Hainaut's