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Original Articles

Cellular and molecular portrait of eleven human glioblastoma cell lines under photon and carbon ion irradiation

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ABSTRACT

This study aimed to examine the cellular and molecular long-term responses of glioblastomas to radiotherapy and hadrontherapy in order to better understand the biological effects of carbon beams in cancer treatment.

Eleven human glioblastoma cell lines, displaying gradual radiosensitivity, were irradiated with photons or carbon ions. Independently of p53 or O^6-methylguanine-DNA methyltransferase[1] status, all cell lines responded to irradiation by a G2/M phase arrest followed by the appearance of mitotic catastrophe, which was concluded by a ceramide-dependent-apoptotic cell death. Statistical analysis demonstrated that: (i) the SF2[2] and the D10[3] values for photon are correlated with that obtained in response to carbon ions; (ii) regardless of the p53, MGMT status, and radiosensitivity, the release of ceramide is associated with the induction of late apoptosis; and (iii) the appearance of polyploid cells after photon irradiation could predict the Relative Biological Efficiency[4] to carbon ions.

This large collection of data should increase our knowledge in glioblastoma radiobiology in order to better understand, and to later individualize, appropriate radiotherapy treatment for patients who are good candidates.

Introduction

The glioblastoma multiform (GBM) is a heterogeneous and highly invasive entity, making it the most aggressive brain tumor. Even for selected patients receiving optimal surgery followed by temozolomide-based chemoradiotherapy according to the EORTC trial 26981/22981-NCIC, survival remains poor, with median survival that does not exceed 15 months [1]. Over the past decade, a variety of different therapeutic strategies have been explored, however, a systemic targeted approach is partially limited by the heterogeneous biology of GBM and the low ability of most conventional cytotoxic drugs to cross the blood–brain barrier [2,3].

Due to a better dose localization in the tumor volume and a high relative biological effect (RBE) for cell killing, hadrontherapy with carbon ions seems to be particularly promising. Because very few hadrontherapy centers exist, the number of biological and clinical studies is limited. The results of a phase I/II clinical trial on patients with GBM combining radiotherapy, chemotherapy, and carbon ion irradiation were promising in terms of progression-free and overall survival with relatively manageable toxicity [4]. Two other clinical trials are currently ongoing in order to better evaluate the efficiency of a carbon ion boost in GBM treatment [5,6].

Several *in vitro* studies have investigated the biological response of GBM cells to photon irradiation. Most of these studies focused on a limited aspect of the biological response and on only 2 or 3 cell lines [7,8]. These limitations are even more highlighted for hadrontherapy because of its novelty and the difficulty of access to platforms [9].

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[1] MGMT: O^6-methylguanine-DNA methyltransferase.
[2] SF2: surviving fraction at 2Gy.
[3] D10: dose for 10% survival.
[4] RBE: Relative Biological Efficiency.

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Therefore, molecular and cellular investigations of GBM are required in order to improve the prediction and treatment of brain tumors. Here, we have examined the radiobiological features of human glioma cell lines following standard or innovative radiotherapy in order to optimize and secure antiglioma strategies. Investigations of the MGMT and p53 status, the radiosensitivity, the type of cell death induced, and the ceramide-dependent apoptosis pathway in a unique collection of 11 GBM cell lines led us to present a cellular and molecular portrait of this type of tumor and provide a strong basis for integrating data into a simulation or mathematical treatment-planning model.

Materials and methods

Cell culture

The eleven GBM cell lines used were a gift from the Pr. Verelle (EA3846, Clermont-Ferrand, France) T98G, SF763, U87MG, SW1783, U373MG, CB193, M059K, FOG, U118, SF767 and U251. Cells were grown as previously described [10].

Irradiation procedure and pharmacological treatment

250 kV photon irradiations were performed on a X-RAD 320 irradiator (PXI) and carbon ion irradiations (72 MeV/u, LET 33.6 keV/µm) at GANIL facilities [11].

Analysis of clonogenic cell survival

Cell survival following irradiation was quantified using a standard colony-forming assay [12].

Cell cycle analysis

The proportion of cells in each phase of the cell cycle was quantified after propidium iodide labeling [12]. Ten thousand events were analyzed using an LSRII-Beckman-Coulter flow cytometer.

Ceramide quantification

The ceramide content was quantified by HPLC with fluorometric detection [13,14].

Cellular morphological characteristics

Cell morphological characteristics were visualized after 4′,6-diamidino-2-phenylindole-dihydrochloride (DAPI) staining. Apoptotic, giant, multinucleated cells, anaphase bridges, and cells containing micronuclei were scored after fluorescence microscopy visualization. A minimum of 500 cells were counted on 2 or 3 slides per experiment.

RT-qPCR of MGMT

After extraction of RNA with an RNeasy Micro kit (Qiagen, Germantown, USA), reverse transcription was performed with a Quantitect Reverse Transcription kit (Qiagen Germantown, USA) as described previously [15]. Then, qPCR was conducted using a Quantitect SYBR green PCR Master Mix (Qiagen) to quantify MGMT expression on a Stratagene Mx3000 system (Agilent Technologies). The geometrical mean of 2 reference genes (GAPDH and β-actin) was used to normalize gene expression.

Statistical analyses

Spearman's rank test and a Wilcoxon paired test were performed using R and Excel software.

Results

Basal molecular status (p53, MGMT) in a collection of 11 glioblastoma cell lines

The p53 status of the 11 GBM cell lines is reported in Table 1, since its expression contributes to radiation-induced apoptosis. MGMT gene expression was also quantified by RT-qPCR since its level can predict the response to temozolomide treatment [16].

In our panel of cells, 7 have a nonfunctional p53 and 7 do not express MGMT. Among all the studied cell lines, only SW1783 is p53 mutated and expressed MGMT, whereas two (SF767, CB193) are p53 wild-type and do not have MGMT expression.

Radiobiological parameters estimated using the clonogenic cell survival assay

The survival fraction at 2 Gy (SF2), the radiation dose required to obtain 10% survival (D10), the α and β values, and the RBE of carbon ions at 10% survival (Dose for 10% survival after photon irradiation/Dose for 10% survival after carbon ion irradiation) were calculated (Table 1). In response to photon irradiation, an important variation in the SF2 values was observed between the cell lines (0.24 for the U251 cells, 0.87 for the T98G cells). Ten of the 11 cell lines studied are radioresistant with an SF2 greater than 0.45. Regarding the D10 and the α/β ratios, the same important range of values was found. After carbon ion irradiation the SF2 decreased in a range from 0.08 (SF767) to 0.38 (SF763). The D10 followed the same trend. As expected, carbon beam is more effective in cell killing than photon since the average RBE was 2.05 ± 0.57. Interestingly, a positive correlation was noted between SF2 values for photon irradiation and SF2 for carbon ions ($P < 0.0001$, $r = 0.9431$), and secondly between D10 values for photons and D10 for carbon ions ($P = 0.0051$, $r = 0.7745$). No significant correlation was found between SF2 for photons and the RBE ($P = 0.79$, $r = 0.09$) nor between the p53 and MGMT combined status and the SF2 for photons or carbon ions.

Cell cycle evolution after photon or carbon ion irradiation

We investigated whether photon or carbon ion irradiation could affect the distribution of cells in the cell cycle (Table 2). At 24 h, cell cycle analysis of non-irradiated cells found that 46%–72% of the cells were in the G1 phase, 7%–18% were in the S phase, and 8%–31% were in the G2/M phase. At the same time, after 10 Gy photon irradiation, a transient arrest in the G2/M phase was found for all the cell lines. For some cells, this arrest persisted for up to 48 h postirradiation.

To compare the biological effects of carbon ion irradiation with photons, the biologically equivalent dose (5 Gy) corresponding to a mean RBE of 2.05 ± 0.57, and not the physical dose (10 Gy), was delivered. Following carbon beam irradiation, a massive G2/M arrest was observed at 24 h and was maintained up to 48 h. The most important G2/M arrest was observed at 24 h in the SF767 cell line and involved 79.9% of the cell population. The percentage observed after carbon ion irradiation was approximately the same.

Cell death by mitotic catastrophe

The possible involvement of mitotic catastrophe [17] was investigated (Table 3) and the appearance of polyploid cells quantified. After photon irradiation, for the majority of cells an increase started at 48 h, whereas for U87MG, U118, or SF767, the number of polyploidy cells only increased at 120 h. After carbon ion irradiation, the appearance of polyploid cells was observable earlier than 48 h for U87MG, SW1783, and FOG cell lines. At 240 h the percentage of polyploid cells decreased without return to control values. To confirm these results, microscopic changes in cells morphology were analyzed. As after flow cytometry analyses, a gradual increase in abnormal cells (anaphase bridges, multinucleated and giant cells) was observed after irradiation, mainly between 48 and 120 h, and decrease at 240 h.

The number of cells containing more than one micronucleus increased from 48 to 120 h after both types of irradiation followed by a decrease until 240 h.

After photon irradiation, the count of abnormal cells was closely correlated to the appearance of polyploidy ($P = 8.57\ 10^{-6}$, $r = 0.53$). This correlation was supported by a negative correlation between