Original Article
Implantation of glioblastoma spheroids into organotypic brain slice cultures as a model for investigating effects of irradiation: a proof of concept

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Abstract: Glioblastoma is the most frequent malignant brain tumor with an overall survival of only 14.6 months. Novel in vitro models preserving both tumor tissue and the interface between tumor and brain tissue are highly needed in order to develop novel efficient therapeutic strategies. Additionally, models for studying the effects of radiotherapy in combination with novel strategies are lacking but important since radiotherapy is the most successful non-surgical treatment of brain tumors. The aim of this study was to establish a glioblastoma spheroid-organotypic rat brain slice culture model comprising both tumors, tumor-brain interface and brain tissue to provide a proof of concept that this model is useful for studying effects of radiotherapy. Organotypic brain slice cultures cultured for 1-2 days or 11-16 days corresponding to immature brain and mature brain respectively were irradiated with doses between 10 and 50 Gy. There was a high uptake of the cell death marker propidium iodide in the immature cultures. In addition, MAP2 expression decreased whereas GFAP expression increased in these cultures suggesting neuronal death and astrogliosis. We therefore proceeded with the mature cultures. Using confocal time-lapse microscopy and detection of tumor cells by immunohistochemistry, tumor cell migration from the spheroids into the slice cultures was revealed, and found to be unaffected by irradiation. However, the expression of the proliferation marker MIB-1 decreased. In conclusion the results showed - as an initial proof of concept - that the established model has potential value in glioblastoma research for developing novel therapeutic strategies comprising irradiation.

Keywords: Brain slice cultures, radiation, short-term, glioblastoma, co-cultures

Introduction

Glioblastoma is the most frequent malignant brain tumor with patients having a median survival of only 14.6 months [1]. The standard treatment includes surgical resection, fractionated radiation and concomitant as well as adjuvant chemotherapy with temozolomide. Radiotherapy is the most successful non-surgical treatment providing a significant survival benefit [2, 3]. Alkylating chemotherapy with temozolomide (TMZ) only has an effect in about half of the patients being dependent on MGMT promotor methylation status [4]. Radiation therapy is therefore a firm part of many novel therapeutic strategies.

In vitro models preserving both tumor tissue, the interface between tumor- and brain tissue and the brain tissue itself are highly needed to develop novel treatment strategies taking also the critical migrating tumor cells as well as the brain tissue itself into account. Organotypic rat brain slice cultures have earlier been used as an in vitro model [5-7] to study neurotoxicological compounds, ischemia and tumor cell migration [8]. These cultures are characterized by preservation of the basic structure and connective organization found in the brain tissue of origin. In most recent studies the brain slices have been cultured between the air-medium interface on semi-porous membranes receiving oxygen from above and medium from below.

Thereby they can be maintained for several weeks to months in culture [9, 10].

Several approaches have earlier been used to study radiosensitivity of glioblastoma cells. Monolayer cultures of glioblastoma cell lines have frequently been used although; it is assumed that spheroid cultures better reflect the actual in situ environment [11, 12]. The spheroids are cultured as three-dimensionel multicellular structures that partly resemble the actual tumor tissue in patients by having close cell to cell contacts. Culturing patient-derived glioblastoma tissue as spheroids in serum-free medium supplemented with growth factors has been shown to enrich for more immature tumor cells with stem-cell properties [13-15]. These stem-like cells have been suggested to be highly resistant against radiotherapy [16], making an approach integrating the use of such spheroids highly clinically relevant.

The aim of the present study was to establish an in vivo-like in vitro model for studying the effects of irradiation by combining brain slice cultures and three-dimensional multicellular spheroids. In this glioblastoma spheroid-organotypic brain slice culture model, glioblastoma spheroids were implanted into the brain slices. First, the effect of radiation on the brain tissue alone was investigated using cortico-striatal brain slices from newborn rats cultured for 1-2 or 11-16 days corresponding to immature brain and mature brain, respectively. Acute cell death was evaluated with the cell death marker propidium iodide (PI) [6, 9, 10] and neuronal degeneration and astrogliosis were detected with the neuronal marker MAP2 and the astrocytic marker GFAP, respectively using immunohistochemical staining [6, 10]. Since the mature brain slice cultures were viable after irradiation in contrast to the immature brain slice cultures, we proceeded with the mature cultures and implanted spheroids into these cultures [5, 6]. Tumor cell migration was monitored by confocal time-lapse microscopy and further investigated by immunohistochemistry. The migration of tumor cells was not compromised by radiation, however a dose-dependent decrease in the proliferation marker MIB-1 was found. Methodologically the results showed-as an initial proof of concept-that the established model has potential value in glioblastoma research for developing novel therapeutic strategies comprising irradiation.

Materials and methods

Preparation of organotypic brain slice culture

The use of animals was approved by The Animal Experiments Inspectorate in Denmark (permission J. No. 2008/561-1572). Organotypic cortico-striatal slice cultures were prepared by the interface method as previously described [6, 17]. Briefly, the cultures were prepared from 0-24 hours old Wistar rat pups (Taconic). The slices were incubated at 36°C in humidified air containing 5% CO_2, and the medium was changed twice a week.

Organotypic brain slice cultures exposed to radiation

Organotypic brain slice cultures were exposed to radiation at 0, 10 or 50 Gy at different time points after preparation and start of culturing (1-2 and 11-16 days). Radiation was carried out at the Laboratory of Radiation Physics at the Department of Oncology, Odense University Hospital. The samples were irradiated on an Elekta Synergy accelerator (Elekta Ltd.) using 6MV photons at gantry angle 180 and field-size 30×30 cm^2, with the samples centered in the field. Two cm perspex provided build-up. The treatment setup was CT-scanned on a Somatom scanner (Siemens AG) and dose calculation performed in Pinnacle (Philips). Cell death was evaluated using PI [6, 18]. PI uptake was visualized using fluorescence microscopy with a standard rhodamine filter and a digital camera (Leica DFC 300 FX). The captured images were analysed using Image J (National Institutes of Health, USA) measuring a mean intensity of the outlined cortex and striatum.

Implantation of spheroids into organotypic brain slice cultures

The Regional Scientific Ethics Committee approved the use of human glioma tissue in the present study (approval number S-20110022). A glioblastoma stem cell-like containing spheroid culture (T76) was established in our laboratory, and cultured in serum-free medium as previously described [19]. Spheroids were labeled with DiI (1,1'-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate), and implanted into brain slice cultures as previously [5]. The day after implantation co-cultures received radiation doses of 0, 10 or 50 Gy, respectively.
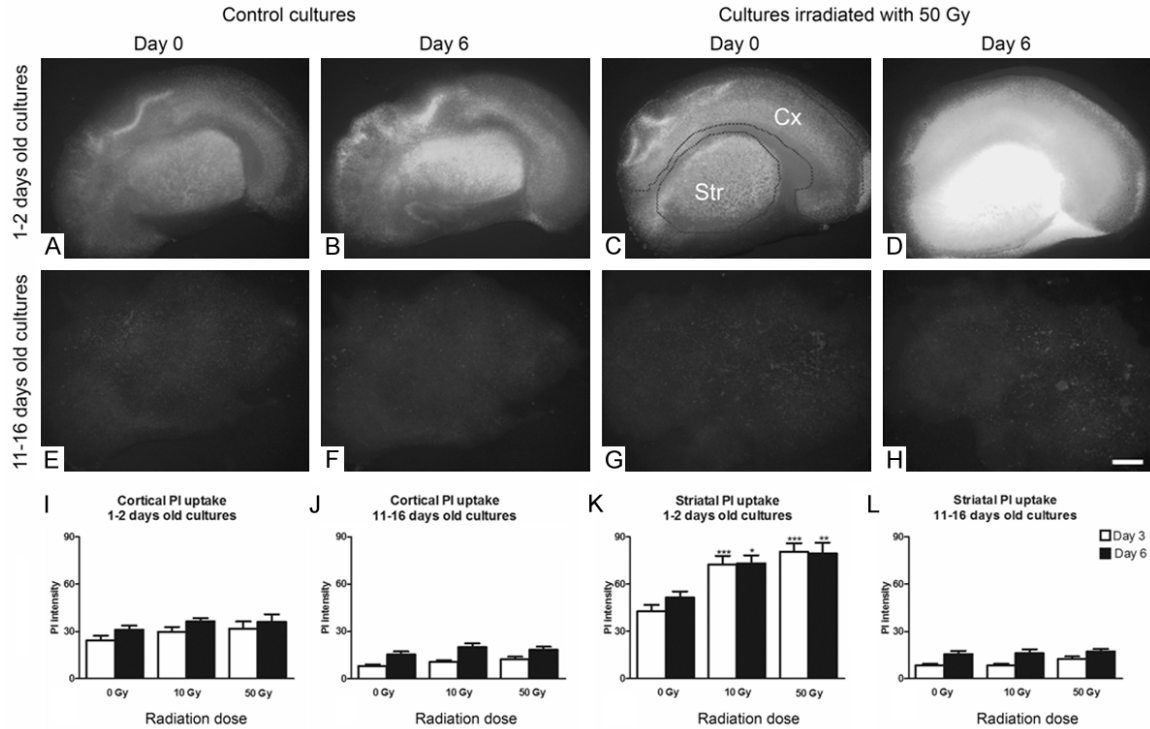
Figure 1. Effects of irradiation on organotypic brain slice cultures prepared 1-2 and 11-16 days prior exposure. Uptake of the cell death marker propidium iodide (PI) was measured in both control cultures (A, B, E, F) and irradiated cultures (C, D, G, H) by outlining cortex and striatum. A high baseline PI uptake was found in cultures prepared 1-2 days before exposure (A, C). However, striatal PI uptake was significantly higher in irradiated cultures (10 and 50 Gy) compared to control cultures (D, K) when cultures were prepared 1-2 days before irradiation. In cultures prepared 11-16 days before irradiation no difference was found in striatal PI uptake (H, L). No difference in cortical PI uptake was found when comparing irradiated cultures and control cultures, neither in 1-2 nor in 11-16 days old cultures (I, J).

For investigation of tumor cell migration into the surrounding brain tissue, confocal laser microscopy was used to obtain z-stacks of images (Nikon Eclipse TE2000-E inverted confocal microscope with perfect focus system). Images were taken prior to radiation and again three and six days after exposure. At day six, the co-cultures were fixated and thereafter sectioned and stained immunohistochemically.

Immunohistochemistry

As previously described [6, 19], three-micrometer sections of paraffin-embedded spheroids were de-paraffinized and heat-induced epitope retrieval was performed in a TEG buffer solution (10 mmol/L Trisbase and 0.5 mmol/L EGTA). After blocking of endogenous peroxidase activity by incubation in 1.5% hydrogen peroxide (H_2O_2), the sections were incubated for 60 minutes with primary antibodies against MAP2 (1+2000), GFAP (1+2000), P53 (1+200), Ki-67 (1+200), MIB-1 (1+200) and Nestin

(1+6000). Detection of antigen-antibody complexes was carried out using the detection system EnVision (Dako), with diaminobenzidine as chromogen. MAP2 staining was quantified using the software NIS-elements AR 3.0 (Nikon).

Statistics

All data were expressed as mean + standard error of mean (SEM). The data was evaluated using a one-way ANOVA test with Bonferroni correction. Statistical significance was defined as *$P<0.05$, **$P<0.01$, ***$P<0.001$.

Results

Cortical and striatal PI uptake after irradiation

In general, PI uptake in cultures prepared 1-2 days before exposure seemed higher compared to cultures prepared 11-16 days before radiation (**Figure 1**). This was seen both at day zero
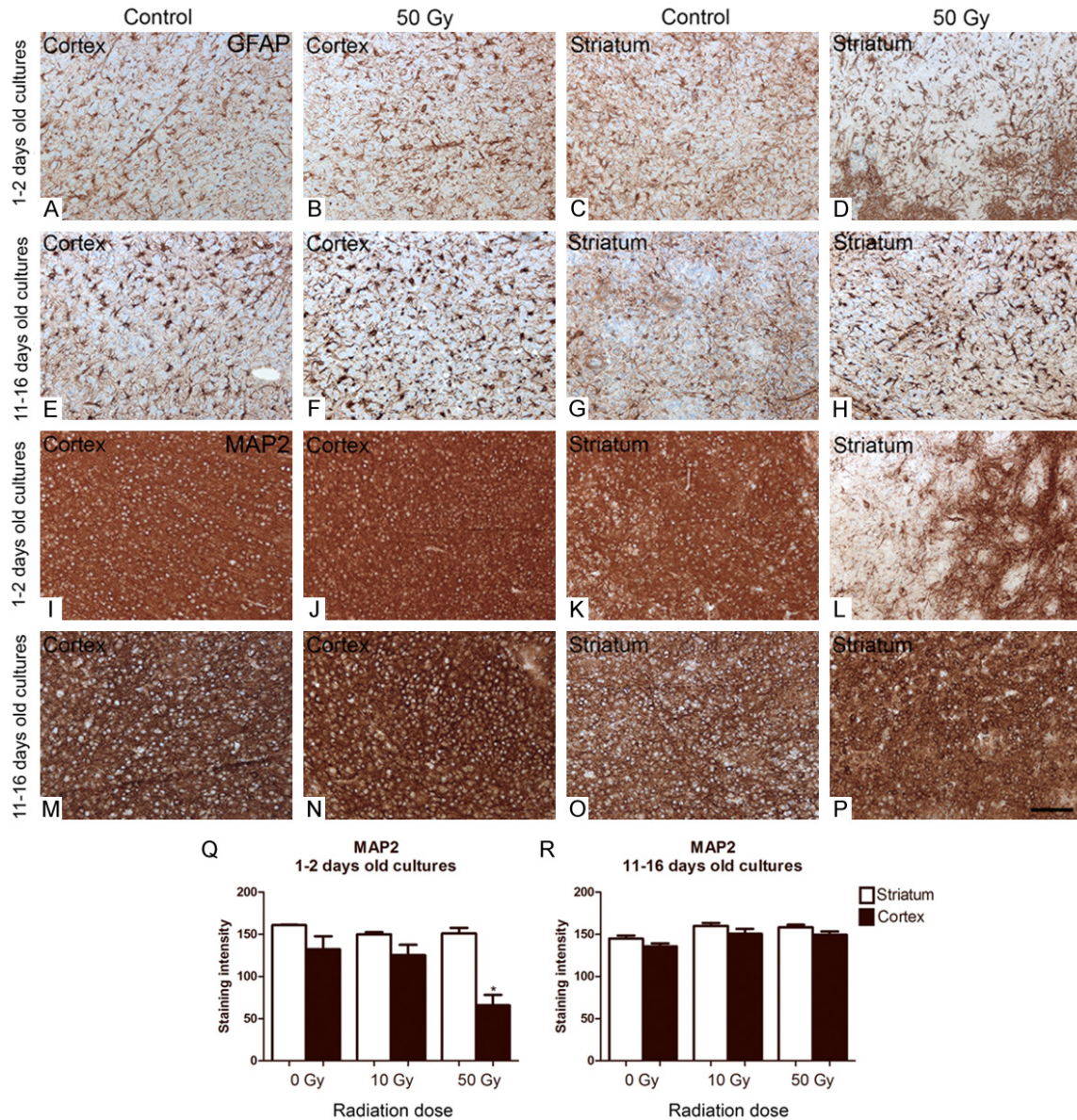
Figure 2. Brain slice cultures were fixed, paraffin embedded, sectioned (3 µm) and immunohistochemically stained for GFAP (A-H) and MAP2 (I-P). Striatum seemed most vulnerable towards radiation, with an increased GFAP expression and a decreased MAP2 expression (C, D, K, L) in cultures prepared 1-2 days before exposure. GFAP and MAP2 showed no difference in staining intensity in cortex using cultures prepared 1-2 days before irradiation (A, B, I, J). No difference in GFAP (E-H) or MAP2 (M-P) in cortex or striatum was seen in cultures prepared 11-16 days before irradiation. Densitometric measurements of MAP2 immunostaining suggested a significantly decreased staining intensity in striatum of cultures prepared 1-2 days before exposure to 50 Gy (Q). No reduction was found in cultures prepared 11-16 days before irradiation (R). Scalebar 100 µm.

and six (**Figure 1A-H**). When measuring the PI uptake no significant increase in cortex was seen in irradiated cultures independent of the duration from preparation of cultures to administration of radiation (**Figure 1I**, **1J**). However, in 1-2 days old cultures a high baseline PI uptake in cortex was seen in both control and irradiated cultures both three and six days after treatment (**Figure 1A-D**, **1I**). Slice cultures irradiated 11-16 days after preparation had a low PI uptake in cortex in both control and irradiated cultures (**Figure 1E-H**, **1J**). PI uptake in striatum was increased in cultures irradiated after 1-2 days with 10 Gy (P<0.05) and 50 Gy (P<0.01), despite a high baseline PI uptake (**Figure 1A-D**, **1K**). Slice cultures irradiated
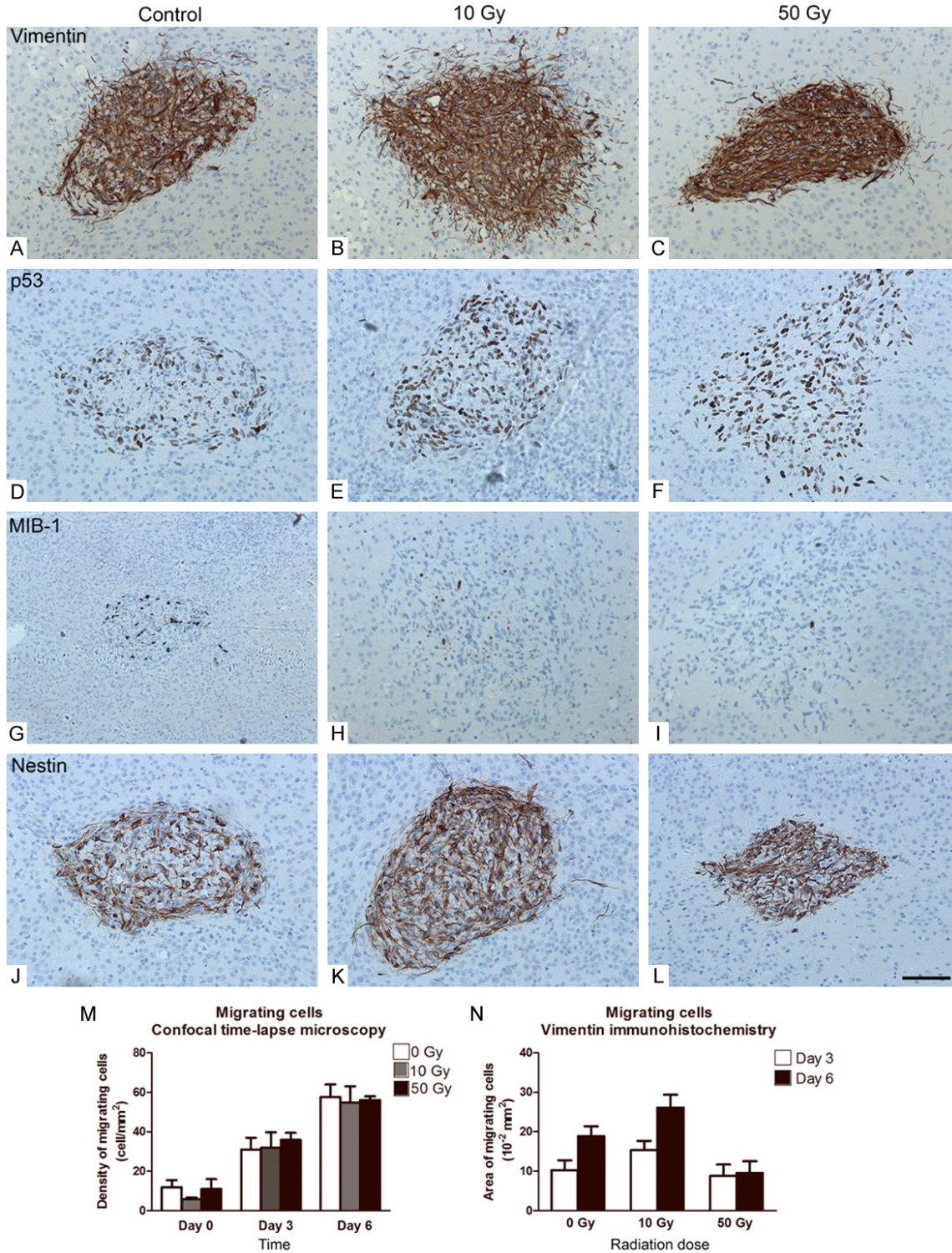
Figure 3. Glioblastoma spheroids were implanted into brain slice cultures and exposed to irradiation with 10 or 50 Gy. Histological sections of co-cultures were immunohistochemically stained with vimentin (A-C), p53 (D-F), MIB-1 (G-I) and Nestin (J-L). The human specific vimentin staining revealed that tumor cells migrated into the brain slice culture even after irradiation (A-C). Expression of p53 was unaffected by radiation (D-F), whereas expression of the proliferation marker MIB-1 decreased in a dose-dependent manner (G-I). Expression of nestin expression was not affected by radiation (J-L). Using confocal time-lapse microscopy, a z-stack of images was taken of the spheroids before radiotherapy and again three and six days after radiotherapy. The z-stacks were accumulated into one im-

age. The density of migrating cells was assessed in a 200 μm zone around the spheroid. There was a pronounced increase in the number of migrating tumor cells with time, but no reduction after irradiation (M). Furthermore, the total spheroid area including the migrating cells was calculated (day three minus day zero and day six minus day zero). Irradiation with 50 Gy appeared to reduce migration but no significant reduction was found (N). Scalebar 100 μm.

after 11-16 days did not show any increase in PI uptake in striatum (**Figure 1G**, **1H**, **1L**).

Expression of GFAP

The expression of GFAP was investigated to detect potential astrogliosis. Control cultures showed presence of astrocytes in both cortex and striatum (**Figure 2A**, **2C**, **2E**, **2G**). In cortex, astrocytes were found not to be affected by irradiation (50 Gy) in cultures prepared 1-2 days before exposure (**Figure 2B**). Interestingly, in certain areas of striatum we observed a high GFAP expression after irradiation (**Figure 2D**). The GFAP staining in both cortex and striatum in cultures prepared 11-16 days before exposure resembled the control cultures (**Figure 2E-H**).

Expression of MAP2

A MAP2 staining was performed to detect potential neuronal damage after irradiation. Intense MAP2 staining was seen in both cortex and striatum in all control cultures (**Figure 2I**, **2K**, **2M**, **2O**). The MAP2 staining intensity in cortex was not affected by irradiation with 50 Gy 1-2 days after preparation (**Figure 2J**). However, a significant decrease in MAP2 staining intensity was observed in striatum of cultures irradiated with 50 Gy 1-2 days after preparation (**Figure 2L**). In cultures prepared 11-16 days prior to irradiation MAP2 staining in the cortex and the striatum was unaffected (**Figure 2N**, **2P**). By densitometric measurements a significant loss of MAP2 staining was seen in striatum irradiated with 50 Gy ($P<0.05$), but only in cultures prepared 1-2 days before irradiation (**Figure 2Q**). No loss of MAP2 staining was found in cultures prepared 11-16 days before exposure neither in cortex nor striatum (**Figure 2R**).

Implantation of spheroids into organotypic brain slice cultures

To investigate the effect of radiation on spheroids and migrating tumor cells, spheroids were implanted into the brain slice cultures. Migrating tumor cells were followed by confocal time-lapse microscopy and thereafter co-cultures were fixed, paraffin embedded and sectioned followed by immunohistochemial staining (**Figure 3**). The spheroids migrated into the surrounding tissue as seen in the vimentin staining (**Figure 3A-C**). The expression of p53 seemed unaffected by radiation (**Figure 3D-F**), whereas expression of the proliferation marker MIB-1 decreased in a dose-dependent manner (**Figure 3G-I**). Nestin was not affected by irradiation (**Figure 3J-L**). Irradiation did not reduce the density of migrating cells detected by confocal time-lapse microscopy, neither three nor six days after exposure (**Figure 3M**). However, the area of invading cells tended to decrease when exposed to 50 Gy both three and six days after exposure, although this was not found to be significant (**Figure 3N**).

Discussion

In this study we established a glioblastoma spheroid-organotypic brain slice culture model comprising both tumors, tumor-brain interface and brain tissue. We demonstrated radiation-induced neuronal death and astrogliosis in immature brain tissue and therefore we used the more mature brain tissue for establishing the co-culture model. Migration of tumor cells from spheroids into the brain slice cultures was not compromised as a result of radiation, but spheroid proliferation decreased as suggested by reduced MIB-1 expression.

In the immature brain slice cultures; there was a high basic PI uptake already before irradiation, suggesting that the cultures need more than two days to recover from the slice preparation process. The striatum seemed especially vulnerable towards irradiation, as suggested by the high striatal PI uptake in immature cultures. Immunohistochemical staining showed that MAP2 expression decreased significantly in the striatum of these cultures, suggesting marked neuronal degeneration. As expected from the PI uptake and the MAP2 staining, GFAP staining showed astrogliosis in the striatum of these cultures. In line with these results previous in vivo studies found that irradiation decreased

neurogenesis in mice 48 h after irradiation [20, 21].

In mature brain slice cultures irradiated 11-16 days after preparation, control cultures showed a low PI uptake. PI uptake in irradiated cultures did not increase and in line with this MAP2 and GFAP expression did not show any signs of neuronal damage or astrocytic reaction, respectively. Based on our results and since most gliomas occur within the adult population, we chose the most mature brain slice cultures for the co-culture experiments.

Immunohistochemical investigations of co-cultures suggested, as expected, a dose-dependent decrease in the spheroids of the proliferation marker MIB-1 in response to radiation. However, neither p53 nor Nestin expression levels were changed in response to radiation. Using time-lapse microscopy, migration of cells into the brain tissue could be closely followed and subsequent immunohistochemical staining confirmed the presence of migrating cells in the brain parenchyma. However, the degree of migration seemed to be unaffected by irradiation. Previously the effect of radiation on migration has been studied in vitro using confluent layers of cells or in vivo using different models [22-24]. The obtained results have been variable possibly being explained by differential radiosensitivity of different cell subpopulation of glioma cells [25].

In contrast to in vitro models with tumor cells growing on a surface our model has the advantage of having a tumor spheroid with tumor cells being located in a three-dimensional environment similar to tumor tissue in a patient. Moreover, tumor cells migrate into a three-dimensional brain tissue and can be followed over time and identified in the tissue. Compared to orthotopic in vivo models [26] our model is much faster and less time consuming but it lacks blood vessels and a functional blood-brain barrier. Another limitation might be the short time span. We followed only tumor cell migration and effects of irradiation over 6 days. However, a previous in vivo study showed that GFAP expression increased three months after radiation of 21 days old mice brains with 5 Gy [20]. The established model may therefore especially be useful for detecting especially acute and pronounced effects and side-effects of novel therapeutical strategies.

In conclusion, the results showed - as an initial proof of concept - that the established model has potential value in glioblastoma research for investigating the effects and potential side-effects of irradiation as well as novel therapeutics strategies combined with irradiation.

Disclosure of conflict of interest

None.

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