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Original Paper

Hypofractionated Radiation Induces a Decrease in Cell Proliferation but No Histological Damage to Organotypic Multicellular Spheroids of Human Glioblastomas

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The aim of this study was to examine the effect of radiation on glioblastoma, using an organotypic multicellular spheroid (OMS) model. Most glioblastoma cell lines are, in contrast to glioblastomas *in vivo*, relatively radiosensitive. This limits the value of using cell lines for studying the radiation effect of glioblastomas. The advantage of OMS is maintenance of the characteristics of the original tumour, which is lost in conventional cell cultures. OMS prepared from four glioblastomas were treated with hypofractionated radiation with a radiobiologically equivalent dose to standard radiation treatment for glioblastoma patients. After treatment, the histology as well as the cell proliferation of the OMS was examined. After radiation, a significant decrease in cell proliferation was found, although no histological damage to the OMS was observed. The modest effects of radiation on the OMS are in agreement with the limited therapeutic value of radiotherapy for glioblastoma patients. Therefore, OMS seems to be a good alternative for cell lines to study the radiobiological effect on glioblastomas. © 1997 Elsevier Science Ltd. All rights reserved.

Key words: organotypic culture, brain neoplasm, glioblastoma, radiotherapy, radioresistance, cell proliferation

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INTRODUCTION

GLIOBLASTOMAS ARE the most common type of primary brain tumour. The prognosis for patients with these tumours is poor. The median survival time, which is approximately 8 months, has not been prolonged significantly despite advances in surgery, imaging techniques, radiotherapy, and the development of various new cytostatic drugs [1–4]. In addition to surgical resection, radiotherapy is considered to be the most effective treatment for glioblastomas [2–5]. However, compared to other solid tumours, glioblastomas are highly radioresistant [5–7]. Several factors such as efficient DNA repair, low oxygen pressure and a high glutathione concentration might contribute to radioresistance [6]. Recently, loss of function of the tumour suppressor pro-

tein p53 has been associated with radioresistance [8, 9]. Accumulation of wild-type p53 in the nucleus can protect cell from radiation-induced DNA damage by the induction of cell cycle arrest or apoptosis [10–12].

The radiation response of glioblastomas *in vitro* has been extensively studied using monolayer cell cultures, whereby cell survival is assessed on the basis of colony formation [13–15]. However, these studies have shown little correlation with the patient's response to radiotherapy: despite the radioresistance of glioblastomas *in vivo*, most glioblastoma cell lines are radiosensitive [6, 7, 13–15]. Recently, an organotypic multicellular spheroid (OMS) model for glioblastomas has been developed, in which most characteristics of the original tumour, such as cellular heterogeneity and glial cyto-architecture, are preserved [16, 17]. In the present study, OMS were used to investigate the effects of radiation on the histology and cell proliferation of the glioblastoma. In addition, changes in p53 expression after radiation were examined.

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MATERIALS AND METHODS

Preparation and culture of OMS

Tumour tissue was obtained at surgery from 8 patients with a glioblastoma multiforme, classified according to the World Health Organization (WHO) [18]. OMS from four glioblastomas were used for hypofractionated radiation treatment. OMS from the remaining four glioblastomas were used for single-dose radiation to determine the changes in p53 expression after radiation. Tissue was collected in Dulbecco's modification of Eagle's medium (DMEM; Flow Laboratories, U.K.). Forty-eight-well plates (Becton Dickinson, Mountain View, California U.S.A) were coated with 0.1 ml 0.75% agarose gel (Sigma, St. Louis, Missouri, U.S.A.) in culture medium, consisting of DMEM supplemented with 10% normal human serum (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB), Amsterdam, The Netherlands), 1 mM glutamine and antibiotics (penicillin and streptomycin, 100 IU/ml) (all from GIBCO, Paisley, U.K.). After the agarose had solidified, 0.3 ml of culture medium was added to each well.

For preparation of the OMS, glioblastoma tissue was processed in the laboratory within 2 h after surgical resection. Blood and necrotic tissue were removed from tumour specimens and fragments of 0.5–1 mm³ were dissected with sterile needles. One fragment was transferred to each well of the agarose-coated plate. The OMS were kept in a tissue culture incubator (98% humidity, 95% air, 5% CO₂) and culture medium was changed once a week. One to three weeks after onset of OMS culture, depending on the time-point of the formation of round-shaped spheroids, the OMS were treated with radiotherapy.

Radiation

Radiation of the OMS was performed using an X-ray generator operating at 250 kV and 15 mA, filtered with 0.5 mm Cu, tube opening of 8 × 8 cm. The dose rate was verified using a cylindrical ionisation chamber (Baldwin and Farmer, type 2570), yielding a dose of 350 cGy per minute. OMS were irradiated in petri dishes (60 mm) containing 3 ml of culture medium at a temperature of 37°C.

Hypofractionated radiation, histology and cell proliferation

OMS from 4 patients with a glioblastoma multiforme (s39, s41, s46, s57) were used. One of the 4 patients (s46) had a recurrence and had received radiotherapy. A total dose of 40 Gy was applied in 8 fractions of 5 Gy given over a course of 2 weeks to the OMS. According to the linear quadratic equation, this fractionation radiation scheme has a radiobiologically equivalent effect on normal brain tissue as 70 Gy in 2 Gy fractions, assuming an α/β value of 2 Gy and no influence of the overall treatment time in the model [19]. Eight OMS from each tumour were treated with this radiation schedule. Control OMS were cultured similarly, but without radiation treatment. After treatment, the OMS were cultured under standard conditions for a further week. Subsequently, the histology and cell proliferation of the OMS were evaluated.

Single-dose radiation and p53 expression

OMS from 4 patients with glioblastoma multiforme (s65, s66, s67, s69) were used. None of these patients had received radiotherapy. A single dose of 50 Gy was given to

the OMS to determine whether glioblastoma cells increase their p53 expression in response to radiation-induced DNA damage. The p53 expression was examined after a single dose of radiation and not after hypofractionated radiation, because it has been reported that p53 expression increases within a few hours of radiation and returns to normal levels 48–72 h later [20]. After treatment, the OMS were cultured under standard culture conditions for 4, 7, 24 h or 8 days. Five OMS of each time period were used. Five control OMS from each tumour were cultured similarly, but without radiation treatment. Subsequently, all OMS were fixed in formalin and imbedded in paraffin. The p53 expression of all OMS was determined by immunohistochemistry on paraffin sections, followed by image analysis. Also, the p53 expression of the resection material from the four glioblastomas was examined immunohistochemically. Immunohistochemistry for the p53 protein was performed with monoclonal antibody, DO-7, which recognises the wild-type and the mutant p53 protein. In addition, the histology and the cell proliferation of all OMS were examined, although not quantified.

Histology

Paraffin section (5 μ m) of OMS were placed on organosilan-coated object slides and dried overnight at 37°C. Paraffin sections stained with haematoxylin and eosin were used to evaluate the histology of the OMS.

Immunohistochemistry

Sections were deparaffinised and endogenous peroxidase activity was blocked in methanol containing 0.3% hydrogen peroxide. After incubation with mono- or polyclonal antibodies (Table 1) for one hour at room temperature, sections were subsequently incubated with either biotin-conjugated rabbit anti-mouse immunoglobulins or biotin-conjugated swine anti-rabbit immunoglobulins. After incubation with a streptavidin-biotin complex (Dakopatts), peroxidase activity was developed in 3,3'-diaminobenzidinetetrachloride (Sigma) with 0.1% hydrogen peroxide. Pepsin treatment was necessary for staining with the F VIII antibody (Table 1) and was performed before blocking of endogenous peroxidase activity. Pepsin treatment consisted of incubation in 0.1 M HCl containing 0.25% pepsin at 37°C for 10 min. Antigen retrieval was performed for staining with the MIB0-1 and DO-7 antibodies (Table 1) after blocking

Table 1. Antibodies (Ab) used in the study

Ab	Specificity	Source	Dilution
GFAP*	glial fibrillary acidic protein;	DAKO	1:1000
F VIII (MAb)	von Willebrand factor;	DAKO	1:40
MIB-1 (MAb)	endothelial cells		
	Ki-67 antigen;	Immunotech	1:100
	proliferating cells		
DO-7 (MAb)	wild-type and mutant p53 protein	DAKO	1:100

Ab, antibodies; *polyclonal antibody; MAb, monoclonal antibody; DAKO, Dakplatts (Glostrup, Denmark); Immunotech, Immunotech SA (Marseille, France).

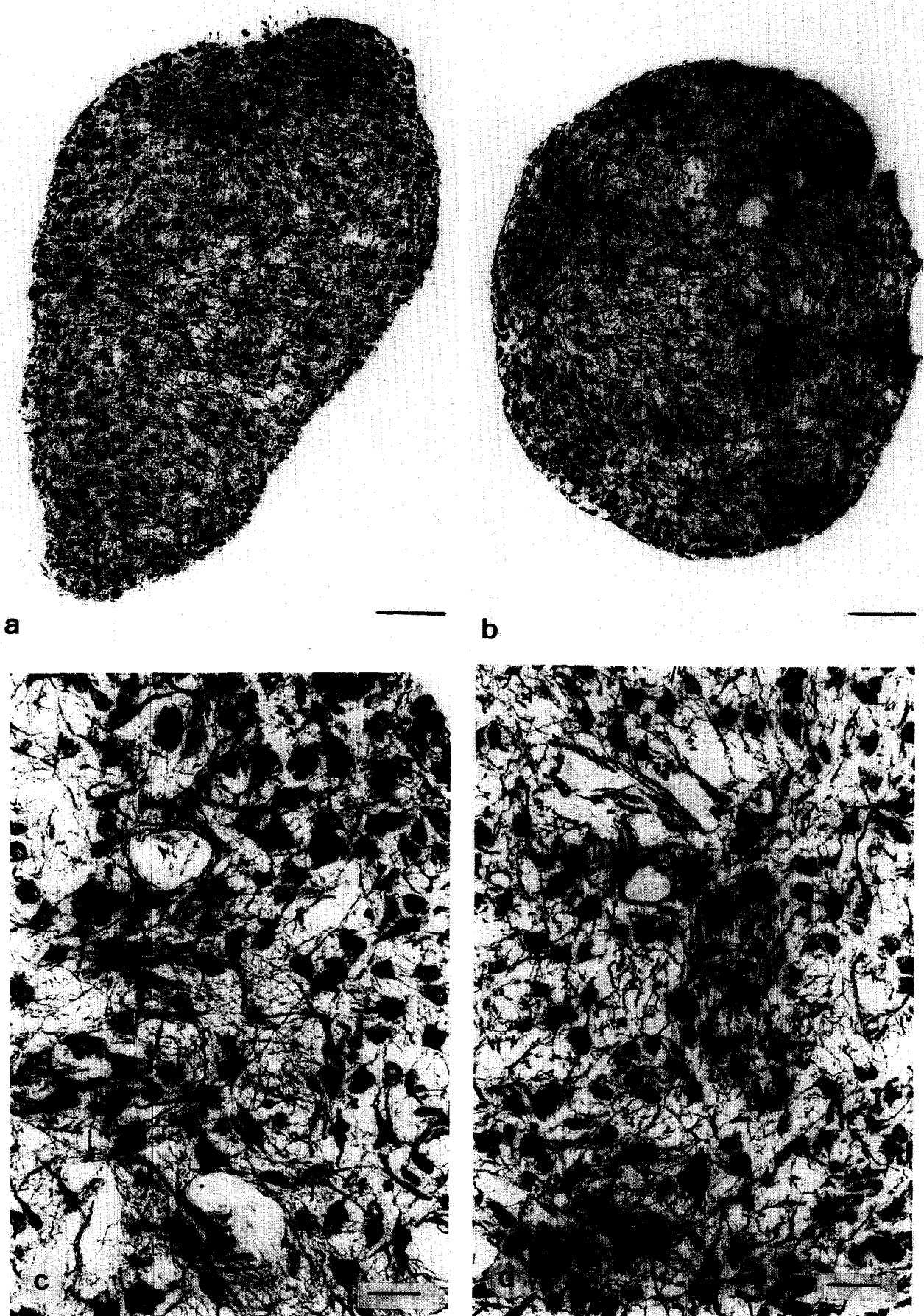


Figure 1. OMS from a glioblastoma multiforme (s57), immunostained for the glial fibrillary acidic protein (GFAP). (a) Untreated control OMS (scale bar = 125 μm). (b) OMS treated by hypofractionated radiation (scale bar = 125 μm). (c) Untreated control OMS (scale bar = 25 μm). (d) OMS treated by hypofractionated radiation (scale bar = 25 μm). No changes in the histology and the glial structure are observed in the OMS after radiation.

the endogenous peroxidase activity. This was carried out by incubation in citrate buffer (2.94 mg/ml trisodium citrate dihydrate in distilled water; pH = 6) at 100°C for 20 min.

Image analysis

The Ki-67 positive nuclei in OMS from the hypofractionated radiation study, and the p53 positive nuclei in OMS from the single-dose radiation were further quantified by computer-based image analysis. Overview images of Ki-67 or p53 immunostained OMS were obtained with a Sony CCD video camera that was connected to an Apple Macintosh Quadra 840 AV computer. Individual OMS were assessed with image analysis software, using the public domain NIH Image program. A macro was developed that, after background subtraction, counted all immunostained nuclei above a predetermined density value. Subsequently, the number of positive nuclei per mm² was calculated. The percentage cell proliferation and the p53 positivity of OMS from each tumour was determined. In addition, the relative cell proliferation and p53 positivity was expressed as a percentage of the OMS with the maximum positivity (100%) from that tumour.

Statistics

Statistical analysis of the data was performed using an unpaired two-sample *t*-test after analysis of variance. *P* values < 0.05 were considered significant.

RESULTS

Typical features of glioblastoma multiforme were observed in all OMS from the 8 glioblastoma patients. In all, a dense meshwork of glial fibrillary acidic protein (GFAP) positive cells and fibrils was observed. The presence of capillaries, connective tissue components, mitotic figures and nuclear atypia were common features in the OMS.

Hypofractionated radiation: histology

One week after hypofractionated radiation treatment, the OMS were histologically examined. Compared to control OMS, none of the OMS that had been treated with hypofractionated radiation showed signs of histological damage, such as decrease in cell density or presence of cellular necrosis (karyorrhexis with loss of the nuclear membrane and disintegration of the nucleus into clumps of basophilic material) (Figure 1). Compared to the control OMS, the radiation-treated OMS showed no changes in the glial structure as determined by immunostaining with GFAP (Figure 1). The number of capillaries and the morphology of endothelial cells, identified by the F VIII monoclonal antibody, were unchanged in radiation-treated OMS compared to untreated control OMS.

Hypofractionated radiation: cell proliferation

The cell proliferation in OMS, one week after hypofractionated radiation treatment, is illustrated in Figure 2. The number of proliferating cells was higher in all control OMS compared to the corresponding radiation-treated OMS. In radiation-treated OMS from three tumours (s41, s46, s57), the cell proliferation decreased significantly (*P* < 0.02); by 7–20 fold compared with control OMS (Figure 2). The relative cell-proliferation index is illustrated in Figure 2b,

showing similar results, although the results for tumour s39 were also significant.

Single-dose radiation: histology and cell proliferation

None of the OMS showed major histological damage 4, 7, 24 h or 8 days after 50 Gy single-dose radiation. Some minor histological changes were observed: in several radiation-treated OMS, a few shrunken nuclei were observed. The number of proliferating cells, as determined by Ki-67 antigen immunostaining, was lower in all radiation-treated OMS compared to control OMS.

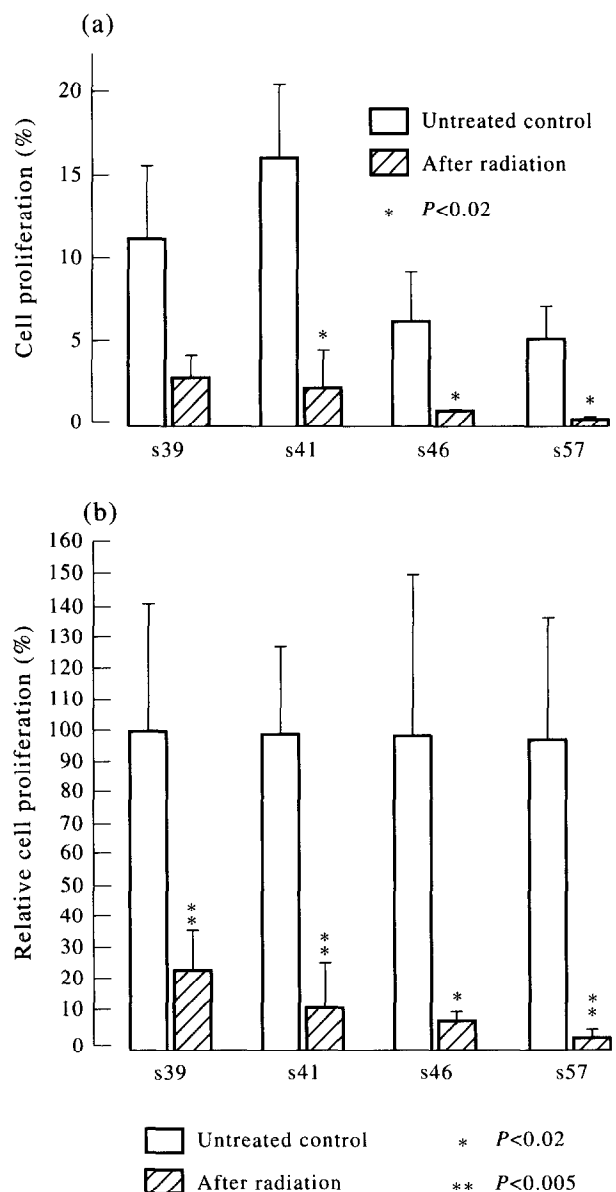


Figure 2. (a) Percentage cell proliferation and (b) relative proliferation index in OMS from four glioblastomas was determined by computer-based analysis after immunostaining for the Ki-67 antigen, one week after hypofractionated radiation treatment: the cell proliferation in the radiation-treated OMS was significantly decreased compared to the untreated control OMS. Error bars represent standard error of the mean.

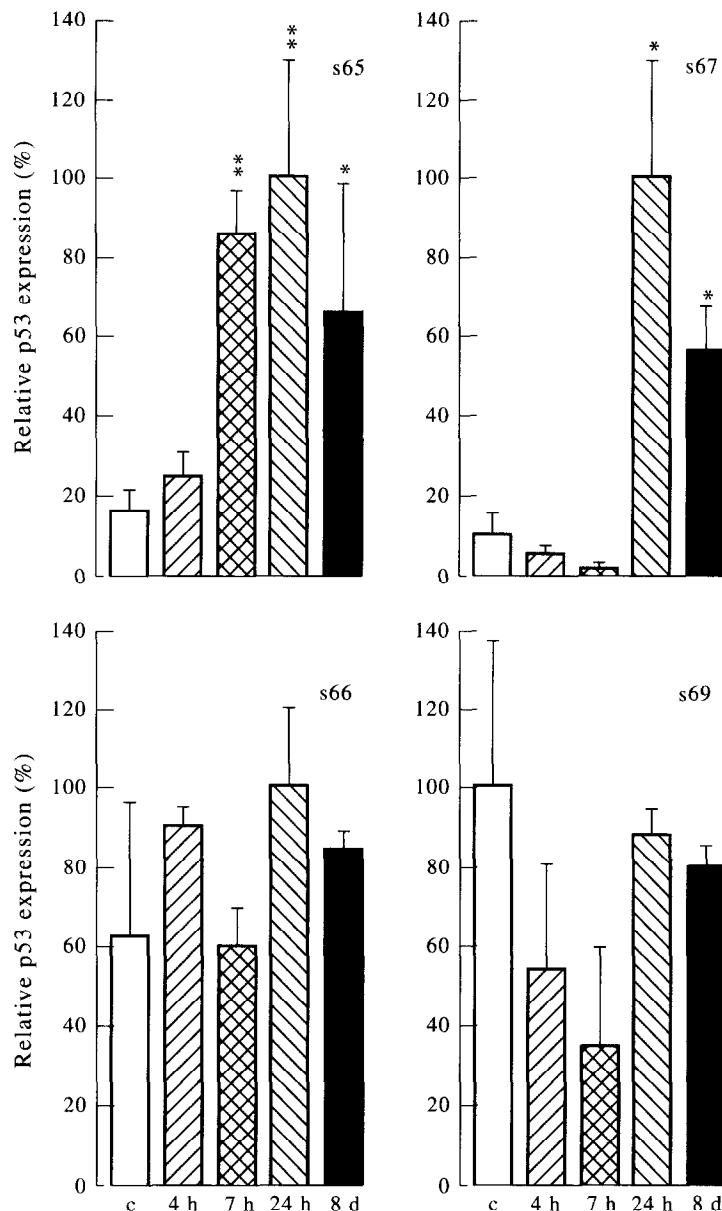


Figure 3. Relative p53 expression was determined by computer-based image analysis after immunostaining for the p53 protein in OMS from four glioblastomas: untreated OMS and OMS treated with a 50 Gy single dose after 4, 7, 24 h or 8 days. * $P < 0.05$, ** $P < 0.005$. Error bars represent standard error of the mean.

Single-dose radiation: p53 expression

p53 expression of the OMS was analysed at an interval of 4, 7, 24 h or 8 days after 50 Gy single-dose radiation and is shown in Figure 3. In the resection material of the four glioblastomas, the overall p53 expression was higher in s67 (30%) and s69 (25%) compared to s65 (5%) and s66 (2%). This was reflected in the untreated control OMS in which p53 positivity was approximately 25% for tumour s67, 15% for tumour s69, 2% for tumour s65 and 3% for tumour s66.

In OMS from two (s65 and s67) tumours, the p53 expression increased significantly ($P < 0.05$) after radiation compared to the corresponding control OMS. Maximum p53 expression was found at 24 h after single-dose radiation. Eight days after radiation, relative p53 expression was still significantly increased in both glioblastomas ($P < 0.05$). In all OMS, the nuclei that were p53 positive were not

shrunk or fragmented. In OMS from the remaining two tumours (s66, s69), p53 expression did not change significantly after radiation compared to the corresponding control OMS.

DISCUSSION

Although glioblastoma cell lines have often been used to study the radiation response of glioblastomas, in contrast to glioblastomas *in vivo*, most glioblastoma cell lines are relatively radiosensitive [6, 7, 13]. Several properties of glioblastoma cell cultures may explain this discrepancy. First, a cell line represents only a small subpopulation of the original tumour due to selection during culture: the cellular heterogeneity of the original tumour is lost with increasing *in vitro* passage. Second, a cell line is an exponentially growing population. When exposed to radiation most cells are in the M or G2 phase, which are the most sensitive phases for

radiotherapy [21]—in contrast, gliomas *in vivo* display cell cycle asynchrony. Third, trypsinisation, which is required when glioblastoma cell lines reach maximum density, damages the cell membranes which might sensitise the tumour cells to radiation. In addition, spheroids prepared from glioblastoma cell lines have been used to study the radiation effects on glioblastomas [22, 23]. Such spheroids have a three-dimensional character, display some cell cycle heterogeneity, and have intimate cell–cell contact. However, these spheroids are also treated with the protease trypsin, and they lack the original tumour's structure and heterogeneity. An alternative model is to transplant glioblastoma cell lines as tumour xenografts in animals to study the radiation effects of glioblastomas *in vivo*. Results of these studies have shown that these cell line xenografts are also relatively radiosensitive, which does not correlate with the poor clinical outcome of glioblastoma patients after radiotherapy [24]. OMS might be a more reliable culture system compared to the cell line models, since most characteristics of the original tumour, for example, the cellular heterogeneity and the glial cyto-architecture, are preserved [16, 17].

In the present study, we found that hypofractionated radiation, with a radiobiologically equivalent dose to standard radiation treatment for glioblastoma patients, induced a decrease in tumour cell proliferation in OMS of human glioblastomas, as determined by immunostaining for the Ki-67 antigen (Figure 2). However, hypofractionated radiation did not induce cytolytic damage to the OMS (Figure 1). Even OMS that had received a 50 Gy single dose showed only minor histological damage. The clinical outcome of glioblastoma patients is favourably affected by radiotherapy [2, 3, 5]. Nevertheless, glioblastomas are considered to be one of the most radiation-resistant human tumours [5–7]. The limited effect of radiotherapy for glioblastoma patients is reflected by the modest effect of radiobiological equivalent doses on OMS of glioblastomas. Our results suggest that the limited therapeutic value of radiotherapy for glioblastoma patients might be caused by a decrease in tumour cell proliferation.

In tumour cell lines, loss of the p53 function, as a consequence of mutations in the *TP53* gene, has been associated with intrinsic resistance to radiation [8, 9]. In contrast, increased expression of wild-type (functional) p53 after radiation can protect cells from radiation-induced DNA damage by inducing cell cycle arrest or apoptosis [10–12]. In the present study, OMS from two (s65, s67) glioblastomas showed an increased number of p53 positive nuclei (7 h, 24 h or 8 days) after a 50 Gy single dose of radiation (Figure 3). One of the these tumours had probably lost p53 function due to a mutation in the *TP53* gene: the tumour had approximately 30% p53 positive cells as analysed by immunohistochemistry, and also showed loss of heterozygosity (LOH) of chromosome 17p (data not shown) [25]. Therefore, in this tumour the increased p53 expression after radiation was not functional. The loss of function of the p53 protein might have contributed to the radioresistance of this tumour [8, 9]. In the other tumour (s65) the *TP53* gene was probably intact: the tumour had only 5% p53 positive cells as analysed by immunohistochemistry, and no LOH of chromosome 17p (data not shown) [25]. The p53 positive nuclei in this tumour (s65) were not shrunken or fragmented, indicating that the radiation-induced p53 overexpression had not resulted in cell death through apoptosis

[26]. It might be that the observed p53 overexpression in these OMS (s65) was responsible for the induction of a growth arrest and for the observed decrease in cell proliferation. However, in the OMS of two other tumours, no increased p53 expression was found after radiation, despite the fact that in these OMS a decrease in cell proliferation was observed. This indicates that mechanisms other than p53 upregulation must be responsible for the decreased number of proliferating cells. Since in tumour s65, the p53 protein was probably intact, apart from loss of p53 function [8, 9], other mechanisms are probably involved in radioresistance of glioblastomas, for example, efficient DNA repair and/or a high glutathione concentration [6]. Furthermore, a low oxygen pressure has been related to radioresistance. In the (rapidly-growing) spheroids prepared from cell lines, hypoxic regions are present in the centre of the spheroids but in OMS central hypoxic regions are absent (Professor H. Acker, Max Plank Institute, Dortmund, Germany). Therefore, hypoxia cannot explain the radioresistance of glioma OMS.

In conclusion, the modest effects of hypofractionated radiation on OMS are in accordance with the limited therapeutic value of radiotherapy in the treatment of glioblastoma patients. In this respect, OMS resemble glioblastomas *in vivo* more closely than the frequently used cell culture models, and OMS may be a good culture model to study the underlying mechanisms of radioresistance. Furthermore, OMS might be useful for studying how to improve the radiation response of glioblastomas, for example, by using radiosensitisers.

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