

Combined modality therapy of gemcitabine and irradiation on human glioma spheroids derived from cell lines and biopsy tissue

FABIAN FEHLAUER^{1,2,3}, MARTINA MUENCH¹, ERNST J. SMID³, BEN SLOTMAN³,
ECKHARD RICHTER¹, PAUL VAN DER VALK² and PETER SMINIA³

¹Department of Radiation Oncology, UKL, University of Luebeck, Germany; Departments of ²Pathology
and ³Radiation Oncology, Academic Hospital Vrije Universiteit Amsterdam, The Netherlands

Received June 30, 2005; Accepted August 23, 2005

Abstract. Multicellular tumor spheroids have been used to examine aspects of combined modality treatment since they often recreate the *in vivo* tumor environment much more closely than other models. The radioenhancement by gemcitabine (dFdC) on human glioma spheroids derived from cell lines (CLS) and biopsy tissue, grown as organotypic multicellular spheroids (OMS), was studied. CLS of GaMg and U87 and OMS of four glioblastoma patients were used. Radiochemosensitivity was determined using migration and proliferation assays on CLS. In OMS, histology and immunohistochemical studies of MIB-1, p53, and p21 expression were examined 24 and 48 h following treatment. Cell death (ethidium homodimer) was studied using a fluorescence cell viability assay. In CLS, combination treatment led to migration inhibition in GaMg and U87 of 85% and 62% (dFdC 46% and 52%, RT 21% and 43%) and proliferation inhibition of 83% and 85%, respectively. Following dFdC + RT in OMS (% of cases), apoptosis and p21 expression increased (50%), p53 expression increased (75%) and cell proliferation decreased (75%). Only minor morphological damage was observed. Confocal laser scanning microscopy identified an increased dead cell core after dFdC + RT (50%). In conclusion, dFdC can lead to an additively radioenhancement in CLS and individual OMS.

Introduction

Malignant gliomas are among the most radioresistant tumors. Although radiotherapy following surgical resection is the most effective treatment, survival in these patients is unsatisfactory. The median survival is only 10-12 months for glioblastoma multiforme (1). Radiotherapy is considered the

most effective adjuvant therapy to surgery, but has reached a plateau in its effectiveness. The modest increase in survival time after radiotherapy treatment has been ascribed to the high intrinsic resistance of gliomas to X-irradiation. To improve survival in patients with malignant glial tumors, novel concepts are required. Further approaches include chemotherapeutic agents as an adjuvant modality or in combination with radiation as a radiosensitizer. Several different culture models have been used to determine the intrinsic radiosensitivity of gliomas. These include monolayer cultures of glioma lines, both early and late passage after initial isolation, and spheroids derived from these cell lines known as cell line spheroids (reviewed in ref. 2). Another culture system used to study gliomas *in vitro* is the organotypic multicellular spheroid (OMS) (3). It is assumed that spheroid cultures can better predict the *in vivo* response compared to monolayer cultures, since cell-cell contact, variation in cell cycle, altered metabolism, and diffusion of nutrients or drugs may influence the outcome (4-6). Gemcitabine (2,2-difluoro deoxycytidine, dFdC) is a promising cytostatic drug. Gemcitabine (dFdC) is a nucleoside analog of cytidine with significant cytotoxicity and a radiosensitizing effect on solid tumor cells *in vitro* and *in vivo*. Clinically, dFdC as a single agent has shown activity in various solid tumors, including NSCLC, small cell lung cancer, head and neck squamous cell cancer, germ cell tumors, and tumors of the bladder, breast, ovary, cervix, pancreas, and biliary tract, as well as some hematologic malignancies (7). Phase II clinical trials on glioblastoma multiforme, which studied the effect of gemcitabine as a first-line therapy before radiation or as a salvage therapy at first relapse (8,9) did not demonstrate a survival improvement.

Two *in vitro* studies on cultured malignant glioma cell lines (10,11) and one animal study on Fisher rats bearing 9L glioma (12) have reported cytotoxic effects of dFdC. These experiments were done without irradiation. Another *in vitro* study determined a radioenhancement by dFdC on a glioma cell line and corresponding cell line spheroids (13). Until now, the radiosensitizing effect of gemcitabine has not been evaluated clinically. In this study, we used two human glioma cell lines cultured as spheroids and organotypic multicellular spheroids (OMS) of four glioblastoma patients to determine the radiosensitizing effect of gemcitabine.

Correspondence to: Dr Fabian Fehlaue, Department of Radiation Oncology, Universitätsklinikum Eppendorf, University of Hamburg, Martinistr. 52, 20254 Hamburg, Germany
E-mail: fehlaue@uke.uni-hamburg.de

Key words: glioma, irradiation, gemcitabine, spheroids

Materials and methods

Monolayer cell culture. Two human glioma cell lines were used for multicellular tumor spheroid cultures. The GaMg cell line was obtained from a 42-year-old female with histologically proven GBM (14). The human U87Mg cell line was kindly supplied by Dr J. Carlsson, Uppsala University, Uppsala, Sweden. Both cell lines were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, UK) supplemented with 10% fetal calf serum, 2 mM L-glutamine and 50 μ g/ml gentamycin (Gibco BRL) in 25 cm² flasks in a 37°C, 5% CO₂, 95% humidified air incubator.

Cell line spheroids. Spheroid cultures of GaMg were formed with the liquid-overlay technique (15). In short, exponentially growing monolayers were trypsinized, and 5x10⁶ cells were seeded in 20 ml of growth medium into 80-cm² agar-coated tissue culture flasks. After a 10-day culture, spheroids with a diameter between 200 and 250 μ m were selected for the experiments. These spheroids do not present central necrosis and have few hypoxic cells (16,17).

Organotypic multicellular spheroids. Fresh tumor tissue was obtained during surgery (open resection) from six patients with a primary glioblastoma, as classified according to the World Health Organization (WHO). The specimens were taken from tumor areas corresponding to regions with contrast enhancement on preoperative computerized tomography scans and transferred in Dulbecco's modified Eagle's medium (DMEM; Flow Laboratories, Scotland).

For preparation of the spheroids, glioma tissue was processed in the laboratory within 2 h of resection. Representative tumor tissue was selected based on both a preliminary frozen section histopathological diagnosis made by the responsible neuropathologist and the macroscopic appearance. Blood and necrotic tissue were removed from tumor specimens, and fragments of 0.5-1.0 mm³ were dissected with sterile 21G2 0.8x50 needles. The 48-well plates were coated with 100 μ l of a semisolid agarose gel consisting of a 1:1 mixture of 1.5% agarose and DMEM supplemented with 4 mM L-glutamine, 100 μ g/ml gentamicin (all from Gibco BRL) and 20% heat-inactivated human serum (Bio-Whittaker, MD, USA). After the agarose had gelled, 300 μ l of culture medium consisting of DMEM supplemented with 2 mM L-glutamine, 100 μ g/ml gentamicin and 10% heat-inactivated human serum was added to each well.

One biopsy fragment was transferred to each well of the 48-well plates. The spheroids were kept in a standard tissue culture incubator (98% humidity, 5% CO₂, 37°C) and the culture medium was changed once a week. Cultured tissue fragments were studied twice a week with a phase-contrast microscope, and fragments that became spherical within the first week were used for further study. Only tissue from 4 patients with a fraction of fragments that formed spheroids of >40% were included in this study. Two weeks after the onset of cultures, the biopsy spheroids were treated.

Irradiation. Due to the differences in response to irradiation, which was tested previously (data not shown), a 20 Gy single dose RT for OMS, 10 Gy single dose RT for GaMg cell line

spheroids, and 5 Gy single dose RT for U87 cell line spheroids were performed using an orthovolt X-ray generator operating at 250 kV and 15 mA, with a 0.5 mm-thick Cu filter and tube opening of 8x8 cm. Spheroids were irradiated in Petri dishes (60 mm) containing 3 ml of culture medium at 37°C. Previous experiments explored these RT dosages to be effective without sterilizing the spheroids (18).

Gemcitabine treatment. Gemcitabine (Gemzar®), 2,2-difluoro deoxycytidine [dFdC] was kindly provided by the Netherlands branch of Eli Lilly and Co. (Indianapolis, IN, USA). Stock solutions of 1 mM dFdC in PBS were prepared and kept at -20°C, then diluted with medium to appropriate concentrations at the start of incubation. The dose-response experiments for cell line spheroids in this study and another (13) were performed to find an optimal sublethal concentration, which does not permit growth, for further experiments (data not shown). For experiments combining gemcitabine with irradiation, CLSs were incubated with doses ranging from 0.5 μ mol to 0.05 mmol dFdC for 24 h. OMSs were incubated with 0.01 mmol dFdC for 24 h. At the end of gemcitabine incubation, the medium containing drugs was removed, cells or spheroids were washed with PBS, and fresh medium was added. At this point, spheroids were transferred to a fresh agarose-coated 48-well culture plate and irradiated. Following treatment, spheroids were kept in culture. Six treated spheroids from each of the initial 3 treatment groups (dFdC, RT, and dFdC + RT) and six control spheroids were fixed in 4% neutral-buffered formaldehyde solution at 24 and 48 h post-irradiation.

Migration of cell line spheroids (migration assay). Following treatment, cell line spheroids were placed individually into uncoated 16-mm multi-well dishes, which were filled with 1 ml DMEM. Each treatment group consisted of six spheroids, and one group was used as the control. Spheroids were plated within 2 h, and cellular outgrowth from the spheroid was defined as a colony. Two orthogonal colony diameters were measured daily over a 4-day period (96 h) with a phase contrast microscope, and the migratory capacities of glioma cells were then determined by calculating the colony areas from diameter measurements.

Proliferation of cell line spheroids (growth delay assay). For experiments, GaMg and U87 spheroids were individually placed into 16-mm multiwell dishes (Nunc), which were base coated with 0.5 ml 0.75% DMEM-agar and filled with 1 ml of DMEM. Six spheroids were selected from each study group (dFdC, RT, and dFdC + RT), and an additional group was used as the control. After treatment, the dishes were incubated at 37°C. Diameters of the spheroids were measured 2 times a week with a phase contrast microscope over a 15-day period, and the spheroid volume was calculated (volume = $4/3 \pi r^3$).

The culture medium was changed once a week. In order to measure growth delay, the average time required to reach 10 times the initial spheroid volume was determined. Growth delay was defined as the delayed time (days) of treated samples to reach the 5-fold volume of the corresponding control samples.

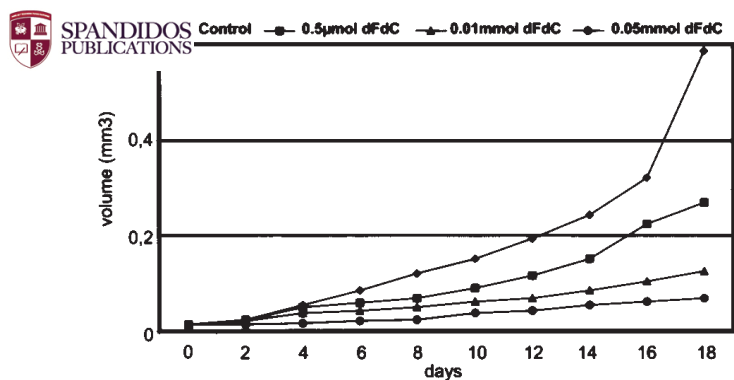


Figure 1. Volume growth (proliferation assay) in GaMg spheroids following different dFdC concentrations.

Morphology of organotypic multicellular spheroids. Paraffin sections (5 μ m) of spheroids were placed on organosilan-coated (3-aminopropyltriethoxysilane; Sigma, USA) object slides and dried overnight at 37°C. Paraffin sections of the original tumor were similarly processed. Histology of the spheroids was evaluated in hematoxylin-eosin-stained sections by light microscopy; the same sections were also used for the assessment of apoptosis. Clusters of intensely basophilic nuclear fragments or an intensely basophilic, shrunk nucleus was construed as morphologic evidence of the apoptotic process. The apoptotic index was defined as the percentage of apoptotic tumor cells, identified as 'apoptotic bodies' (19).

MIB-1, p53, and p21 protein expression of OMS (immunohistochemistry). Monoclonal antibodies were used for immunohistochemistry (MIB-1, p53, and p21), using the avidin-biotin peroxidase complex (ABC) technique according to the manufacturer's protocol (MIB1 and Ki67 protein from Immunotech; DO7 and wild-type and mutant p53 from Dako; p21 and p21 protein from PharMingen).

Briefly, after 5- μ m sections underwent dewaxing and rehydration, endogenous peroxidase activity was blocked for 30 min in methanol containing 0.3% hydrogen peroxide. Antigen retrieval was performed for all monoclonal antibodies by incubating slides in a citrate buffer solution (2.94 mg/ml trisodium citrate dihydrate in distilled water; pH 6.0) in a microwave oven at 180°C for 15 min. Thereafter, the slides were preincubated with normal rabbit serum in a 1:50 dilution in phosphate-buffered saline with 1% bovine serum albumine (PBS/BSA) for 10 min. Subsequently, sections were incubated with monoclonal antibodies (overnight, 4°C), and incubated with biotin-conjugated rabbit anti-mouse immunoglobulin or biotin-conjugated swine anti-rabbit immunoglobulin (all from Dako, Denmark). After incubation with streptavidin-biotinylated horseradish peroxidase complex (dilution 1:200; Dako), peroxidase activity was developed using 3,3-diaminobenzidine-tetrachloride (Sigma) in 0.1% hydrogen peroxide as a chromogen. All sections were lightly counterstained (20 sec) with hematoxylin, dehydrated, and mounted. The signal for p21 was enhanced by the catalyzed reporter deposition technique. Positive control sections from known immunopositive tumors were included for all antibodies. Negative controls consisted of sections of each group, whereby the primary antibody step was omitted.

The evaluation was assessed with a conventional light microscope. For p53, p21 and proliferation activity (MIB-1), all identifiable nuclear staining was regarded as positive. The labeling index was defined as the percentage of immunopositive tumor cells in the spheroids. No endothelial or hematogenous cells were included in the counts. The relative apoptosis count and p53, p21 and MIB-1 positivity of spheroids from each tumor was expressed as the percentage of spheroids with maximum positivity (100%). The multiple factor of protein expression of treatment groups compared to the controls was calculated for each treatment group; a difference within the treated groups of a multiple factor >1 was defined as a marked treatment effect. Statistical comparison of the data was performed using an unpaired sample t-test after analysis of variance. P-values <0.05 were considered significant.

Confocal laser scanning microscopy of OMS (live/dead[®] assay). Viability tests were performed with an MRC-100 confocal laser microscope (Bio-Rad, Hemel Hempstead, UK) 24 h after the experiments. Vital spheroids of all 3 treatment groups and control spheroids were stained with a live/dead viability/cytotoxicity assay (Molecular Probes, Leiden, The Netherlands) according to the manufacturer's protocol. Before staining, the spheroids were twice washed in a PBS bath (Petri dish) and transferred (one per well) into a 96-well plate (uncoated). Surplus PBS was carefully removed, and the spheroids were resuspended in 50 μ l of solution from the live/dead kit (2.0 μ l calcein AM and 4.0 μ l ethidium homodimer-1/1 ml culture medium).

The incubation period was 30 min, preferably at 37°C. Polyanionic cell-permeant nonfluorescent calcein AM is taken up by the living cell and intracellularly processed by esterases into a fluorescent calcein producing an intense green signal (emission spectrum, 495-515 nm). Ethidium homodimer (EtHd) enters cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence upon binding to nucleic acids, thereby producing a bright red signal (emission spectrum, 495-635 nm). EtHd is impermeable to intact cell membranes and can therefore be used to identify single dead cells with intact DNA at an early stage of cell membrane damage. When green and red signals overlap, a dark yellowish signal with less green signals appears on the screen. The spheroid core was optically sectioned (20 μ m thickness) and semi-quantitatively analyzed: +, <10% yellowish/red signal; ++, 10-50% yellowish/red signal; +++, >50% yellowish/red signal. Confocal laser microscopy images were processed using Adobe Photoshop (version 5.0).

Results

Cell line spheroids (CLS)

Proliferation assay. First, proliferation capacity of different dFdC concentrations on spheroids of both cell lines was determined. For GaMg spheroids, a significant reduction of 55% in spheroid growth was observed following 0.5 μ mol dFdC (specific growth delay, SGD 1.5), 80% for 0.01 mmol dFdC (SGD 2.1), and 89% for 0.05 mmol dFdC (SGD 3.6) on day 18 (p<0.05; Fig. 1). For U87 spheroids, a significant decrease of 68% in spheroid volume was observed following incubation with 0.5 μ mol dFdC (specific growth delay, SGD

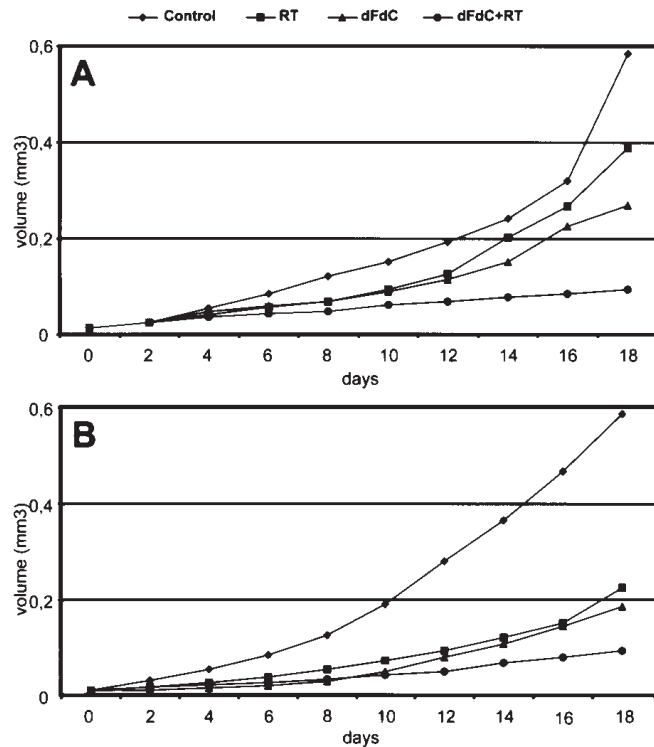


Figure 2. Volume growth (proliferation assay) in GaMg spheroids following 0.5 μ mol dFdC and 10 Gy irradiation (A), and U87 spheroids following 0.5 μ mol dFdC and 5 Gy irradiation (B).

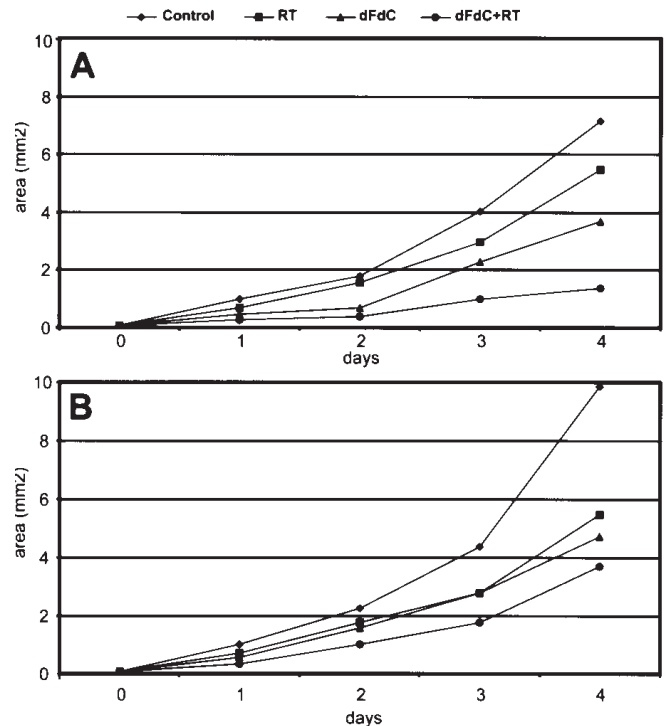


Figure 3. Tumor cell migration (migration assay) from GaMg spheroids following 0.5 μ mol dFdC and 10 Gy irradiation (A), and U87 spheroids following 0.5 μ mol dFdC and 5 Gy irradiation (B).

1.9), 83% with 0.01 mmol dFdC (SGD 2.8), and 90% with 0.05 mmol dFdC (SGD 4.5) on day 18 ($p < 0.05$; Fig. 2B). Based on these experiments, a concentration of 0.5 μ mol dFdC allowing a suitable regrowth capacity was chosen for the migration assay and combined modality experiments of CLS and OMS.

Following RT alone, GaMg and U87 spheroids showed a significant growth reduction of 17% (SGD 1.1) and 63% (SGD 1.7), respectively. Following combined modality treatment, GaMg and U87 spheroids showed an additively significant growth reduction of 83% (SGD 3.3) and 85% (SGD 3.5), respectively (Fig. 2A and B). Thus, the calculated drug enhancement ratio (DER) was 2.2 for GaMg spheroids and 1.8 for U87 spheroids.

Migration assay. The directional cell migration from the spheroids was determined for treated and untreated spheroids. For GaMg spheroids, the tumor outgrowth area was reduced by 46% (dFdC), 21% (RT), and 85% (dFdC + RT) during the 4-day treatment (Fig. 3A). For U87 spheroids, the tumor outgrowth area was reduced by 52% (dFdC), 43% (RT), and 62% (dFdC + RT) during the 4-day treatment (Fig. 3B). When compared to single modality treatment, combination therapy of spheroids from both cell lines led to a significant difference in tumor outgrowth reduction ($p < 0.05$).

Organotypic multicellular spheroids (OMS)

Spheroid culture and volume measurements. OMS established from 4 of 6 tumor samples could be included in this study (S 36, S 38, S 40, and S 41). Phase contrast microscopy showed that approximately 65% of all tumor fragments prepared from fresh tissue formed spheroids within 1 week of culture.

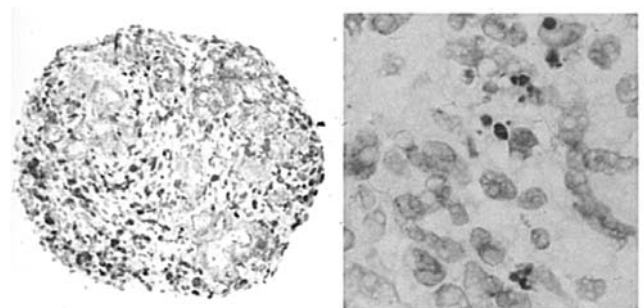


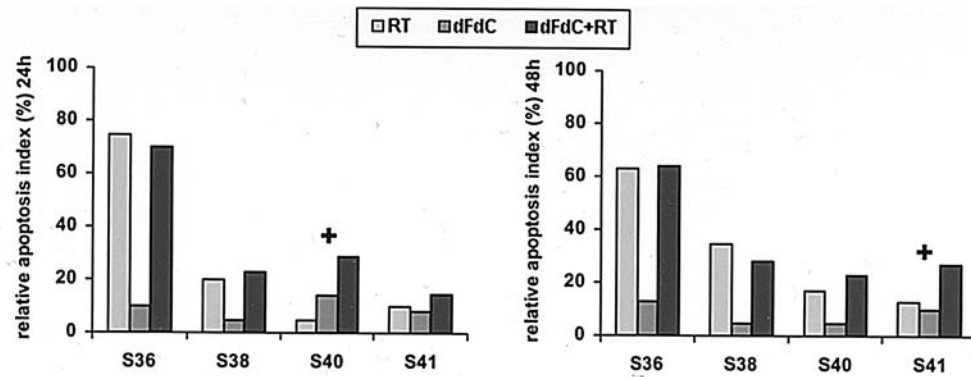
Figure 4. A typical H&E-stained organotypic multicellular spheroid (OMS) showing the increased rate of apoptotic bodies 24 h after combination therapy. Original magnification, x40 (right). Example of elevated immunohistochemical p53 expression in OMS following dFdC and irradiation. Original magnification, x10 (left).

Fragments of 2 patients could not be used for further experiments because single cells and clumps were shed from the fragments without spheroid formation (S 37 and S 39).

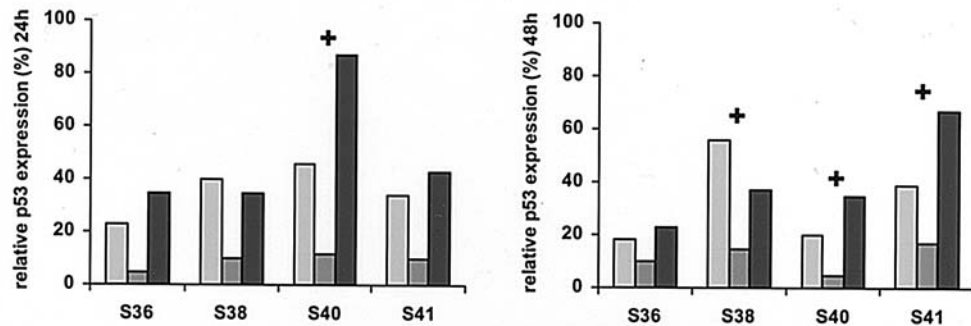
During the culture period and following treatment, the volume of spheroids from three tumors remained steady. Spheroids from one tumor showed a decrease in volume, which was more pronounced 48 h after combination treatment, but not statistically significant from controls.

Morphology and apoptosis. Histologically, the spheroids closely resemble the original individual tumor. As in the tumor *in vivo*, OMS contained tumor cells, endothelial cells and connective tissue. Typical features of glioblastoma were observed in untreated and treated spheroids, including increased cellularity, nuclear atypia, mitotic figures and non-functional capillaries, of which a few showed vascular proliferation.

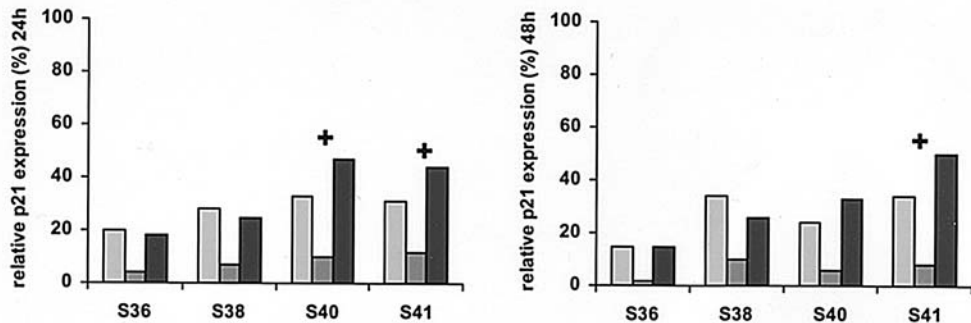
A



B



C



D

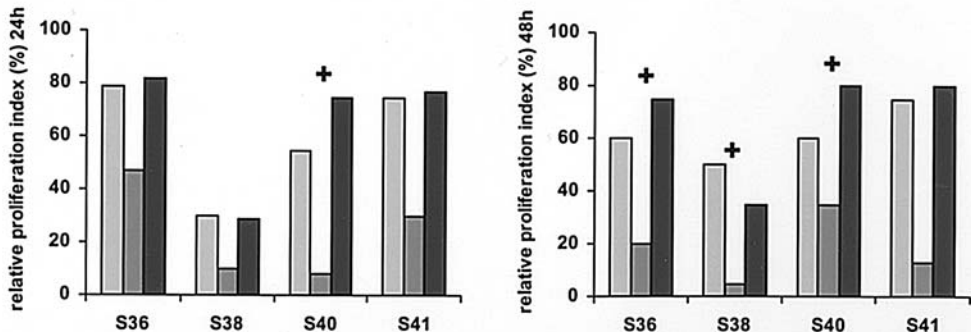


Figure 5. Apoptosis and immunohistochemical expression of spheroids as determined 24 h (left column) and 48 h (right column) following treatment. From top, relative apoptosis index (A), relative expression of p53 (B), relative expression of p21 (C) and relative cell proliferation (D). +, $p < 0.05$.

Neither necrosis, nor perinecrotic pseudo-palisading cellular components were observed.

Compared to the control spheroids, no major morphological damage could be observed in any of the treated spheroids up to 48 h after treatment. No decrease in cell density or changes in the glial structure were seen. Capillaries were preserved in control and treated spheroids at both follow-up intervals.

The number of apoptotic tumor cells increased in spheroids at 24 and 48 h after treatment. Apoptosis, identified as clusters of intensely basophilic nuclear fragments, were seen in both irradiated and control spheroids from all groups (Fig. 4).

Following gemcitabine and RT, individual tumors showed a maximal enhancement of apoptotic bodies in 2 of 4 cases at both time points, and a marked effect was seen in 1

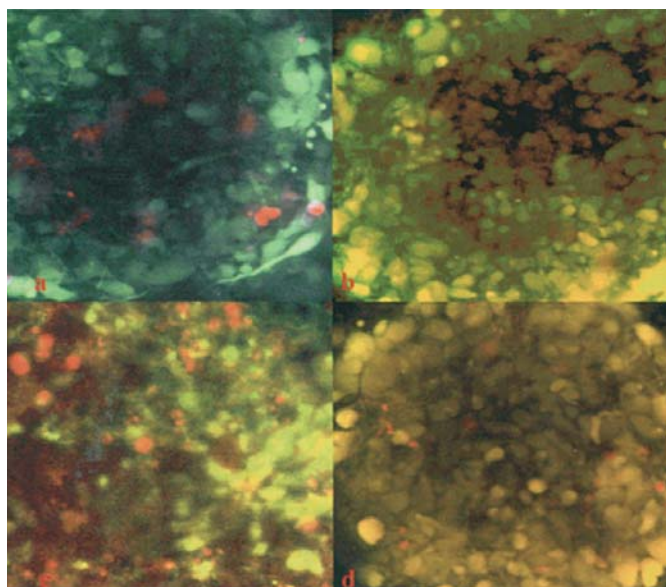


Figure 6. Optical section of a 500- μ m organotypic multicellular spheroid (OMS) performed by confocal laser microscopy showing distribution of ethidium homodimer (EtHd) dead cell staining on the spheroid rim following treatment. Staining was performed in vital spheroids 24 h post-treatment. (a) Example of an untreated OMS as control. Homogeneously green signals of ethidium, as an indicator of living cells, with single red signals of EtHd. (b) Example of a dFdC-treated OMS, which shows green signals and an increased yellowish signal due to the overlap of red and green signals. (c) Example of an irradiated spheroid with an increased yellowish/red signal. Green signals were present in single modality-treated OMS. (d) Section of a multimodality-treated OMS. A more homogeneously distributed yellowish signal was seen, indicating the highest amount of dead cells in the spheroid rim.

of 4 cases (S 40) at the 24 h interval, and 1 of 4 cases (S 41) at the 48 h interval (Fig. 5). Radiation alone was the most effective treatment modality in 2 of 4 cases (24 h for S 36 and 48 h for S 38), as the effects were only slightly more pronounced compared to other treatment groups. Overall, gemcitabine + RT-treated spheroids showed the highest increase of apoptotic fragments, but the effects were not significant.

Cell proliferation. MIB-1 immunopositivity was exclusively nuclear. Control spheroids showed a remarkably wide range of MIB-1 tumor cells (2-30%), which were mostly centered in the peripheral zone. The number of proliferating cells was lower in spheroids of treated groups compared to controls (Fig. 5).

Following gemcitabine and RT on individual tumors, one case showed a maximal decreased proliferation labeling index 24 h later, although the effect was minor compared to other treatment groups. At the 48 h interval, combination treatment led to a decreased MIB-1 labeling index in 3 of 4 cases, and a marked effect was only seen in spheroids of tumor S 40. Overall, gemcitabine + RT-treated spheroids showed the greatest decrease in the MIB-1 index, but the effects were not significant.

p53 expression. In resected material from the original GBM, the overall p53 expression was <5%. In spheroids

established from these tumors, the absolute percentage ranged between 2-7% and immunopositivity was exclusively nuclear. A wide range of percentages of p53 immunoreactive tumor cells was documented between spheroids within the same group.

Following gemcitabine and RT, individual spheroids showed a maximal enhancement of p53 expression in 3 of 4 cases at various time points, and a marked effect was seen in 1 of 4 cases (S 40) at the 24 h interval, and 1 of 4 cases (S 41) at the 48 h interval (Fig. 4). RT alone was the most effective treatment modality in 2 of 4 cases (both S 38 and S 40 at 48 h), and the effects were only slightly more pronounced compared to other treatment groups (Fig. 5). Overall, a significant increase could be detected following RT alone and gemcitabine + RT treatment (both $p < 0.05$).

p21 expression. All original tumors contained <5% p21 immunoreactive tumor cells. In untreated spheroids, the mean p21 expression was 1-2%.

Following gemcitabine and RT, individual tumors showed a maximal enhancement of p21 expression in 2 of 4 cases at the 24 h interval (S 36 and S 40), and 2 of 4 cases at the 48 h interval (S 36 and S 41). In all of these cases, a marked effect was detected. RT alone was the most effective modality with a marked effect in 1 of 4 cases at 24 h following treatment (S 38; Fig. 5). Overall, p21 protein expression increased significantly following RT alone and CDDP + RT treatment ($p < 0.05$).

Confocal laser scanning microscopy (for OMS). Spheroids of all patients showed an extended fluorescent-stained core, with central parts of the spheroids remaining unstained due to limited laser light and limited calceine/ethidium homodimer penetration. Section scans could be performed up to 120 μ m from the surface of spheroids, which allows the ability to distinguish single dead cells within the peripheral rim 24 h following treatment (Fig. 6). In untreated spheroids, single ethidium-stained cells in the peripheral vital cell rim were observed (3-5% of total cell mass) as a phenomenon of physiological cell shedding. In gemcitabine-treated spheroids, a larger amount of lethal-stained cells was detected in the peripheral spheroid rim (10-30% of total cell mass). Due to overlapping green and red signals, cells appeared yellowish. When treated with RT alone and in combination with gemcitabine, a more homogeneous distribution of a yellowish signal, indicating an equal number of red and green cells, was seen in spheroids of 2 patients (>50% of total cell mass). A pronounced yellowish cell signal was present in the 2 patients following combination therapy, but not single modality treatment.

Discussion

In glioma research, four different *in vitro* culture systems are often used. These include characterized permanent cell lines grown as monolayer, monolayer cultures of single cells with an early or late passage number after initial isolation of uncharacterized cell suspensions, three-dimensional tissue cultures as spheroids derived from a cell line known as cell line spheroids (CLS), and spheroids derived from fresh



sue known as organotypic multicellular spheroids. However, most experimental data on the response of glioblastoma to irradiation are based on monolayer cultures.

The major advantages of cell lines are they can be easily obtained, cultured, treated and analyzed with conventional endpoints, such as clonogenic assay. Furthermore, the control and treated samples have the same initial characteristics. Thus, any effects can be attributed to treatment.

The radiosensitivity of glioma cells as determined in monolayer cultures varies widely as reviewed by Taghian *et al* (2). The SF2 values range from 0.12 to 0.87. The high radioresistance observed for these tumors in the clinic cannot be explained by those data or the data from our laboratory (20). Tumorigenesis is a multistep process in which initial cells are transformed into highly malignant derivatives due to genetic changes (21). Even with all of these genetic changes that primarily lead to a growth advantage, not all cells from the tumor will grow *in vitro*; as such, monolayer cultures still represent a better selection compared to cells from the original tumor.

Another but rarely used culture system is the OMS, which is not selected for immortality. OMS maintain original tumor heterogeneity, as well as tumor architecture including tumor vessels and extracellular matrix on a genetic level (3). These OMS are subcultured from fresh surgical tumor material and have been shown to be highly radioresistant (19,22). Hypofractionated radiation of 40 Gy in eight fractions of 5 Gy resulted in a significant decrease in cell proliferation with no histological damage, and even OMS treated with 20 to 50 Gy in a single dose did not show major histological damage.

A more commonly used model system is the CLS. CLS have the advantage of being relatively easy to obtain and maintain in culture compared to OMS. They lack the cellular heterogeneity of OMS, which are derived from a single immortal cell line, but several studies have shown that these cell line spheroids retain some characteristics of the original tumor, which include aspects of tumor morphology and behavior (23,24). In the CLS, as in OMS, cell-cell contact, variation in cell cycle distribution, diffusion effects, altered metabolism, and hypoxia are presumed to influence the outcome of radiation treatment (25,26). Thus, both culture systems of spheroids were used to mimic the *in vivo* situation more accurately than monolayers.

For GBM, early tumor proliferation and migration in terms of recurrence remain the major clinical problem. Both applied assays for CLS reflect the radiochemosensitivity and curability. The proliferation assay determines the growth delay as the function of surviving cells and re-growth rate, while the migration assay determines the outgrowth and migration capacity of glioma cells following treatment. OMS cultures were used to detect the individual treatment response on a histological level, and immunohistochemical protein expression of MIB-1, p53 and p21. In addition, the live/dead assay, rarely used in pre-clinical radioenhancement experiments, was applied to detect cell viability following combined modality treatment via confocal laser microscopy.

In the present study, gemcitabine (dFdC) and irradiation treatment (RT) was more effective in CLS. Combination

treatment led to a migration inhibition in GaMg and U87 of 85% and 62% (dFdC 46% and 52%, RT 21% and 43%) and proliferation inhibition of 83% and 85% (dFdC 50% and 72%, RT 17% and 63%), respectively. The OMS behavior varied widely, which reflects the high heterogeneity of GBM. The apoptosis rate and p21 expression increased 50%, and the p53 expression increased 75% and proliferating cells decreased 75% in OMS following combination therapy. Moreover, not all tumor biopsies gave rise to suitable OMS, and large differences were observed in culture capacity, which may be dependent on the initial genetic differences in these cells and led to malignancy.

Genc *et al* found smaller CLS to be substantially more resistant to radiation than the monolayer cultures, with a dose modification factor of 2.5 (13). This is probably due to factors characteristic for spheroids, such as the presence of hypoxic cells and a high proportion of non-proliferating cells. Since hypoxia was first observed by Sutherland and Durand (4), it has been observed in many types of spheroids (27). As hypoxia is thought to be a major factor responsible for radioresistance of tumor cells (28), it is assumed that hypoxic regions in spheroids influence the effects of radiation. 'Radiobiological' hypoxia was observed in older studies (29,30). However, in more recent studies, the correlation between radioresistance and hypoxia was not evident (27,31,32), suggesting that other factors such as cell-cell contact and the fraction of non-proliferating cells might play a more important role in radiation outcome. As CLS grow, the number of proliferating cells decreases and the number of quiescent cells increases. Due to laboratory conditions, the volume of OMS remains unchanged in long-term culture, which can be attributed to a balance between cell proliferation and cell shedding. In both systems, proliferation becomes limited to the outer rim of the spheroid (5,15,19). However, most studies on the *in vitro* effect of dFdC use monolayer cell cultures, and the mechanism of action was described elsewhere (reviewed in ref. 33).

In addition to its cytotoxic effect, dFdC is a potent radiosensitizer (34,35). The mechanism of radiosensitization or radioenhancement by gemcitabine has been studied by many authors on cell lines of various tumors. Depending on the cell line tested, drug concentration, schedule of administration, and cell proliferation status (e.g. plateau vs. exponentially growth), dose enhancement ratios in the range of 1.1-3.0 have been reported. Our results are in line with previous findings since the drug enhancement ratio (DER) was 2.2 for GaMg spheroids and 1.8 for U87 spheroids.

dFdC radiosensitization has been associated with depletion of the dATP pools, accumulation of cells in S phase of the cell cycle, p53 status independent action, apoptosis induction, and the characterization of intracellular reactions such as an increase of DNA double-strand breaks and modification of repair pathways (7).

Our results show that OMS spheroids are resistant in terms of morphological changes and protein expression of MIB-1, p53, and p21 to dFdC. This is probably due to the fact that the OMS volume remains stable in culture and contains more cells in insensitive cell cycle phases than CLS. In general, the effect of gemcitabine in spheroids may be diffusion limited. The results of Neshateh-Riz *et al* with

radiolabelled deoxyuridine (125IudR) support this view (36). These authors showed a decreased incorporation of 125IudR in glioma spheroids as the spheroid size increases. Maximal incorporation was observed at the proliferating outer rim, while the incorporation decreased with depth.

The cytotoxic action of gemcitabine is largely S-phase-dependent and may thus explain the observation that no clinical benefit was observed in phase II clinical GBM trials (8,9). In those studies, gemcitabine was delivered without concomitant radiotherapy.

It is likely that the glioblastoma tumors *in situ* have a large population of non-proliferating cells, and G0 and G1 cells (37). This will severely hamper the direct cytotoxic action, as well as the radiosensitization of gemcitabine.

In summary, we showed that gemcitabine can enhance the effects of irradiation in both CLS and OMS, while the effect was more pronounced in CLS. Proliferating CLS, with an increase in volume over time, showed a obvious radio-enhancement by dFdC. No obvious changes in volume or histological damage was seen in OMS, while heterogeneous effects on protein expression level (MIB-1, p53, and p21) were observed. Our finding supports the view that cell cycle distribution is important in radiosensitization by gemcitabine and apoptosis is a possible lethal pathway in GBM cells. The results presented in this study also have clinical implications. The cell cycle dependency of gemcitabine obviously reduces the effectiveness of this drug in tumors with a larger population of non-proliferating cells, as in OMS. The optimal radioenhancing effect of gemcitabine may be achieved in rapidly growing tumors, i.e. locally delivered in glioma tumors in combination with radiotherapy after 'debulking' surgery, where it is known that local recurrences grow rapidly.

Acknowledgements

The author (F.F.) was supported by a grant (FE 515/1-1) from the Deutsche Forschungsgemeinschaft (DFG, Bonn, Germany) and European Cancer Centre (ECC, Amsterdam, The Netherlands). M. Münch was supported by the Kreitz-Siftung (Bad Segeberg, Germany).

References

- Gonzalez-Gonzalez D and Hulshof MCM: Neurooncology. In: Primary Tumors and Neurological Complications of Cancer. Radiotherapy. Twijnstra A, Keyser A and Ongerboer de Visser BW (eds). Elsevier, Amsterdam, pp134-158, 1993.
- Taghian A, Ramsay J and Allalunis-Turner J: Intrinsic radiation sensitivity may not be the major determinant of the poor clinical outcome of glioblastoma multiforme. *Int J Radiat Oncol Biol Phys* 25: 243-249, 1993.
- Bjerkvig R, Tonnesen A, Laerum OD, *et al*: Multicellular tumor spheroids from human gliomas maintained in organ culture. *J Neurosurg* 72: 463-475, 1990.
- Sutherland RM and Durand RE: Hypoxic cells in an *in vitro* tumor model. *Int J Radiat Biol Relat Stud Phys Chem Med* 23: 235-246, 1973.
- Sutherland RM: Cell and environment interactions in tumor microregions: the multicellular spheroid model. *Science* 240: 177-184, 1988.
- Kunz-Schughart LA: Multicellular tumor spheroids: intermediates between monolayer culture and *in vivo* tumor. *Cell Biol Int* 23: 157-161, 1999.
- Pauwels B, Korst AE, Lardon F, *et al*: Combined modality therapy of gemcitabine and radiation. *Oncologist* 10: 34-51, 2005.
- Gertler SZ, MacDonald D, Goodyear M, *et al*: NCIC-CTG phase II study of gemcitabine in patients with malignant glioma. *Ann Oncol* 11: 315-318, 2000.
- Weller M, Streffer J, Wick W, *et al*: Preirradiation gemcitabine chemotherapy for newly diagnosed glioblastoma. *Cancer* 91: 423-427, 2001.
- Rieger J, Durka S, Streffer J, *et al*: Gemcitabine cytotoxicity of human malignant glioma cells: modulation by antioxidants, BCL-2 and dexamethasone. *Eur J Pharmacol* 365: 301-308, 1999.
- Ostruszka LJ and Shewach DS: The role of cell cycle progression in radiosensitization by 2',2'-difluoro-2'-deoxycytidine. *Cancer Res* 60: 6080-6088, 2000.
- Degen JW, Walbridge S, Vortmeyer AO, *et al*: Safety and efficacy of convection-enhanced delivery of gemcitabine or carboplatin in a malignant glioma model in rats. *J Neurosurg* 99: 893-898, 2003.
- Genc M, Castro Kreder N, Barten-van Rijbroek A, *et al*: Enhancement of effects of irradiation by gemcitabine in a glioblastoma cell line and cell line spheroids. *J Cancer Res Clin Oncol* 130: 45-51, 2004.
- Akslen LA, Andersen KJ and Bjerkvig R: Characteristics of human and rat glioma cells grown in a defined medium. *Anticancer Res* 8: 797-803, 1988.
- Carlsson J, Nilsson K, Westermark B, *et al*: Formation and growth of multicellular spheroids of human origin. *Int J Cancer* 31: 523-533, 1983.
- Mueller-Klieser WF, Freyer JP, Sutherland RM: Influence of glucose and oxygen supply conditions on the oxygenation of multicellular spheroids. *Br J Cancer* 53: 345-253, 1986.
- Sutherland R, Freyer J, Mueller-Klieser W, *et al*: Cellular growth and metabolic adaptations to nutrient stress environments in tumor microregions. *Int J Radiat Oncol Biol Phys* 12: 611-615, 1986.
- Fehlauer F, Stalpers LJ, Panayiotides J, *et al*: Effect of single dose irradiation on human glioblastoma spheroids *in vitro*. *Oncol Rep* 11: 477-485, 2004.
- Kerr JFK, Wyllie AH and Currie AH: Apoptosis, a basic biological phenomenon with wider implications in tissue kinetics. *Br J Cancer* 26: 239-245, 1972.
- Fehlauer F, Barten-Van Rijbroek AD, Stalpers LJ, Leenstra S, Lindeman J, Tjahja I, Troost D, Wolbers JG, van der Valk P and Sminia P: Additive cytotoxic effect of cisplatin and X-irradiation on human glioma cell cultures derived from biopsy tissue. *J Cancer Res Clin Oncol* 126: 711-716, 2000.
- Hanahan D and Weinberg RA: The hallmarks of cancer. *Cell* 100: 57-70, 2000.
- Kaaijk P, Troost D, Sminia P, *et al*: Hypofractionated radiation induces a decrease in cell proliferation, but no histological damage to organotypic multicellular spheroids of human glioblastomas. *Eur J Cancer* 33: 645-651, 1997.
- Desoize B, Gimonet D and Jardiller JC: Cell culture as spheroids: an approach to multicellular resistance. *Anticancer Res* 18: 4147-4158, 1998.
- Santini MT, Rainaldi G and Indovina PL: Multicellular tumour spheroids in radiation biology. *Int J Radiat Biol* 75: 787-799, 1999.
- Sutherland RM and Durand RE: Cell contact as a possible contribution to radiation resistance of some tumours. *Br J Radiol* 45: 788-789, 1972.
- Olive PL and Durand RE: Drug and radiation resistance in spheroids: cell contact and kinetics. *Cancer Metastasis Rev* 13: 121-138, 1994.
- Gerlach A and Acker H: pO₂- and pH-gradients in multicellular spheroids and their relationship to cellular metabolism and radiation sensitivity of malignant human tumor cells. *Biochim Biophys Acta* 1227: 105-112, 1994.
- Sutherland RM: Tumor hypoxia and gene expression-implications for malignant progression and therapy. *Acta Oncol* 37: 567-574, 1998.
- Durand R and Biaglow JE: Radiosensitization of hypoxic cells of an *in vitro* tumor model by respiratory inhibitors. *Radiat Res* 69: 359-366, 1977.
- Biaglow JE, Varnes ME, Jacobson B, *et al*: Factors influencing the oxygen consumption and radiation response of cultured mammalian cells. *Adv Exp Med Biol* 159: 347-358, 1983.



SPANDIDOS¹ P, Acker H, Eikesdal HP, Kaaijk P, Enger P, Slotman B, Sjøerksvig R: Oxygenation and response to irradiation of organotypic multicellular spheroids of human glioma. *Anticancer Res* 23: 1461-1466, 2003.

32. Buffa FM, West C, Byrne K, Moore JV and Nahum AE: Radiation response and cure rate of human colon adenocarcinoma spheroids of different size: the significance of hypoxia on tumor control modelling. *Int J Radiat Oncol Biol Phys* 49: 1109-1118, 2001.
33. Storniolo AM, Allerheiligen SR and Pearce HL: Preclinical, pharmacologic, and phase I studies of gemcitabine. *Semin Oncol* 24: 2-7, 1997.
34. Shewach DS and Lawrence TS: Gemcitabine and radiosensitization in human tumor cells. *Invest New Drugs* 14: 257-263, 1996.
35. Lawrence TS, Eisbruch A and Shewach DS: Gemcitabine mediated radiosensitization. *Semin Oncol* 24: 24-28, 1997.
36. Neshasteh-Riz A, Angerson WJ, Reeves JR, Smith G, Rampling R and Mairs RJ: Incorporation of iododeoxyuridine in multicellular glioma spheroids: implications for DNA-targeted radiotherapy using Auger electron emitters. *Br J Cancer* 75: 493-499, 1997.
37. Barendsen GW, Van Bree C and Franken NA: Importance of cell proliferative state and potentially lethal damage repair on radiation effectiveness: implications for combined tumor treatments. *Int J Oncol* 19: 247-256, 2001.