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Nonlinearity in MCF7 Cell Survival Following Exposure to Modulated 6 MV Radiation Fields: Focus on the Dose Gradient Zone

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Abstract

The study of cell survival following exposure to nonuniform radiation fields is taking on particular interest because of the increasing evidence of a nonlinear relationship at low doses. We conducted in vitro experiments using the MCF7 breast cancer cell line. A 2.4 × 2.4 cm^2 square area of a T25 flask was irradiated by a Varian Novalis accelerator delivering 6 MV photons. Cell survival inside the irradiation field, in the dose gradient zone and in the peripheral zone, was determined using a clonogenic assay for different radiation doses at the isocenter. Increased cell survival was observed inside the irradiation area for doses of 2, 10, and 20 Gy when nonirradiated cells were present at the periphery, while the cells at the periphery showed decreased survival compared to controls. Increased survival was also observed at the edge of the dose gradient zone for cells receiving 0.02 to 0.01 Gy when compared with cells at the periphery of the same flask, whatever the isocenter dose. These data are the first to report cell survival in the dose gradient zone. Radiotherapists must be aware of this nonlinearity in dose response.

Keywords

IMRT, bystander effect, dose gradient zone, clonogenic assay, MCF7 breast cancer cell line

Introduction

Breast-conserving treatment is the standard care for early-stage breast cancer. It consists of conservative surgery followed by whole-breast irradiation.[1] Radiation therapy is a mainstay of this conservative approach, which not only leads to a 3-fold reduction in local recurrence and but also improves overall survival. Recently, new techniques have emerged. Among them, intensity-modulated radiation therapy (IMRT) is of interest because it improves dose conformity and minimizes the dose to organs at risk, thus minimizing acute and late toxicity. Intensity-modulated radiation therapy, which typically uses 6 MV photons, also avoids the production of secondary neutrons.[2]

Many studies aiming to establish the cell response to ionizing radiation have been conducted on cell cultures uniformly irradiated with various doses and dose rates.[3] In radiotherapy, it is assumed that cell death is proportional to the absorbed radiation dose.[4] During radiotherapy with IMRT, a dose gradient zone is present next to the radiation field, especially within 5-mm margins. In this area, cells irradiated with high doses are close to cells receiving very low doses. There is increasing evidence of a nonlinear relationship at low doses. Actually, biological effects such as adaptive response, hyper-radiosensitivity, and increased radioresistance have been

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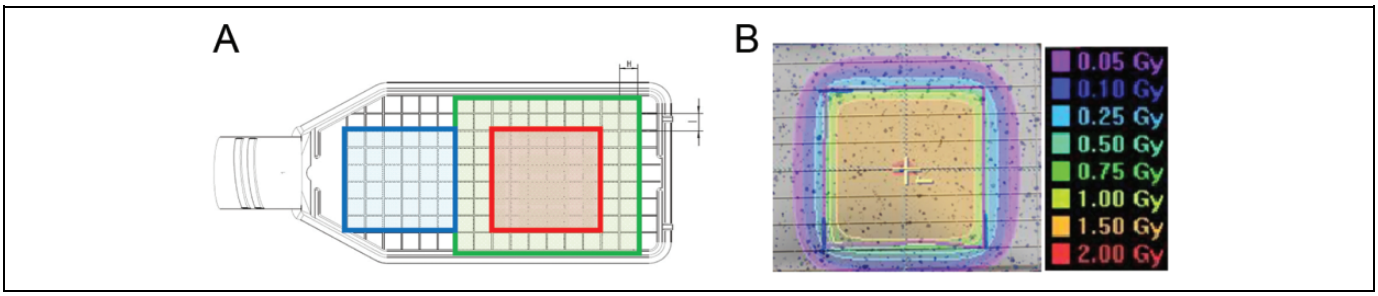

Figure 1. A, Drawing of the T25 culture flask with checkered background and 3 areas: the irradiated field is in red, the out-of-field area in blue, and the dose gradient zone in green. B, Left: Example of isodose distribution as calculated with the Treatment Planning System (TPS) for 2 Gy at the isocenter superimposed with colony forming assay test. Cell colonies are colored dark blue by methylene blue. Right: correspondence of the absorbed dose for each color (only 8 isodoses can be simulated on 1 representation).

described for doses below 0.1 Gy.[5-7] Moreover, bystander effects may interfere with linear response.[8-10] These biological effects could explain the radiotherapy failure with local recurrence observed in about 8% of patients.[11] In order to analyze the cell response to radiation, it is of particular interest to use in vitro models that take both the irradiated cells and those of the peripheral area into account.

Some of these models have been developed to spatially analyze cell survival while taking the surrounding cells into consideration. Responses of cells in the same flask with a localized irradiated area have been evaluated by different methods. Crystal violet optical density can be used for doses above 5 Gy.[12] Blockhuys et al elaborated a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test in order to localize remaining surviving cells but the question of whether it reflects metabolic activity or survival is still under debate.[13] Clonogenic survival assays are the most widely used.[12,14-16] These studies have provided increasing evidence that irradiated and peripheral cells may affect each other's survival or metabolism.

Claridge Mackonis et al exposed various areas of cells to irradiation and found that the cell survival was dependent on the absorbed dose but also on the fate of neighboring cells and was attributed to different bystander effects.[15] Besides the classical bystander effect (type 1), they reported a type 2 corresponding to increased survival of cells communicating with cells receiving a high or lethal radiation dose and a type 3 corresponding to increased survival of high dose irradiated cells when they were in communication with cells receiving a low radiation dose. Butterworth et al also described decreased survival on out-of-field prostate cancer and fibroblast cell lines when cell communication was intact.[17,18] Blockhuys et al irradiated square areas of 1×1 cm^2 and compared cell survival inside and at the frontier of the irradiation area.[13] They found increased cell survival in this peripheral zone. Suchowerska et al showed that cell–cell communication modified cell survival but depended on the radiation dose.[14] These studies provide evidence of a bystander effect existing inside and outside the irradiation field.

The aim of the present study was to compare cell survival inside an irradiation field, in the dose gradient zone on the periphery, and outside the field, using an in vitro model. To date, no data are available on the survival of cells inside the dose gradient zone and at different isodoses. The MCF7 breast cancer cell line was used and a Varian Novalis accelerator, delivering 6 MV photons, provided the irradiation.

Materials and Methods

Cell Line

MCF7 cells were purchased from the American Type Culture Collection (Rockville, Maryland). They were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (Life Technologies, Saint Aubin, France) buffered with 20 mmol/L Hepes, 2 mmol/L glutamine, and 5% fetal bovine serum (FBS) (Sigma-Aldrich, Lyon, France) and incubated at 37°C in a humidified atmosphere containing 5% CO_2. Cells were harvested with trypsin/EDTA (0.05/0.02 v/v).

Clonogenic Assay

In order to localize the irradiation field and the peripheral zone more easily, the cells were seeded in T25 culture flasks with a checkered background. They were grown to mid-logarithmic phase (40%-60% confluence) and fed with fresh medium on the day before the experiments. They were harvested with trypsin/EDTA and then seeded at the low density of 4.000/25-cm^2 flask 18 hours before the irradiation. After irradiation, control and irradiated cells were kept in the same medium until colonies of more than 50 cells appeared. Three culture dishes were used for each point. Then colonies were fixed and stained with 0.3% methylene blue in ethanol. Colonies containing >50 cells were counted with a binocular lens or, in order to allow 3 different persons to perform counting, the flasks were scanned and the images were saved as JPEG files. The surviving fraction was calculated as the ratio of the mean number of irradiated colonies surviving to the mean number of control colonies (control flasks from the same experiment).

This method allowed the superposition of 2 images when the irradiation was performed in a limited area with the accelerator. In these flasks, the surviving fraction was established in 3 areas: inside the irradiated field (2.4×2.4 cm^2, red square) in the peripheral zone (outside the irradiated zone, blue square) and in the dose gradient zone (green square; Figure 1A). The

area of each isodose was calculated using the Treatment Planning System (TPS). Then, the surviving fraction per isodose could be calculated in the dose gradient zone (Figure 1B). Data are presented as the mean ± the standard error of 3 experiments.

Irradiation

Biobeam Cesium irradiator. A BioBeam Cesium irradiator was used to determine the dose-dependent cell survival curve and the Lethal Dose 50 (LD50) of MCF7 cells. A Cesium137 source, of energy 662 KeV, was used. The rate was calculated according to the decay of the source. At the time of the experiments, this was 3.45 Gy/min. Below 4 Gy, absorbers were used in order to reduce the rate. The entire surface of the flasks was irradiated.

Varian Novalis accelerator. T25 culture flasks were irradiated using a 6 MV photon beam produced by a Varian Novalis linear accelerator (LINAC) equipped with a micro-multileaf collimator (Novalis, microMLCm3, BrainLab, IPLAN DOSE 4.0 *TPS*., Munich, Germany). A uniform, 2.4 × 2.4 cm^2, shielded field was created with the micro-MLC. The isocenter and the irradiation field were drawn on the bottom of each flask. The isocenter positioning was performed with laser beams. For irradiation, a 1.5 cm build-up layer of a Phantom (Bolusil®, Kerjean Biotechnologies, Aubergenville, France) was placed under the bottom of the flask and fixed with elastic grips to achieve a stable dose distribution of the cell layer attached to the bottom of the flask. The flasks were placed vertically during irradiation with a gantry angle of 90°. In these geometric conditions, a wet-film of medium covered the cells and the stable dose-distribution to cells was allowed by the bolus. We chose not to fill the flask with culture medium during irradiation in order to keep the same medium after irradiation. Irradiated cells may secrete factors in the medium which can act on neighboring cells in the same flask. This also avoided cells becoming detached during removal of the medium immediately after irradiation. Control flasks were also placed vertically during the irradiation time. For all exposures, unexposed controls were prepared and treated as having undergone sham exposure.

Validation of the experimental design. Before each experiment, ionization chamber measurements were made (Scanditronix/Wellhofer Farmer Type Chamber FC65-P, ionization chamber, vol. 0.65cc, IBA) to verify the nominal LINAC output. The planning was performed with BrainScan (TPS from BrainLab®, IPLAN DOSE 4.0 *TPS*., Munich, Germany). This module calculates the number of monitor units necessary to deliver the required dose. The required dose was applied to the isocenter, and the isodose distributions were expressed as colored fields.

An absolute dose calibration was performed by irradiating EBT3 Gafchromic films with a known dose distribution determined with an ionization chamber. Film calibration sheets were scanned simultaneously with the experimental film sheet 24 hours after the irradiation on an Epson 10000XL. The orientation of the films on the scanner was constant. The dose delivered was 2 Gy on the central axis at the plane of the film (coincident with the plane of the cells). Figure 2 shows the line profiles along the central axis.

Statistical Analysis

Three replicates were counted for each dose region in each experiment to assess the survival fraction. The data are presented as mean ± standard error in all cases. Significance was assessed using the nonparametric Mann and Whitney test.

Results

Survival Curves

In order to assess the LD50, a survival curve was determined using the BioBeam irradiator. The data are presented in Figure 3. At 2 Gy, the surviving fraction was 47%. The lethal dose was 10 Gy. We noted a marked decrease in cell survival at 6 Gy, with less than 1% of live cells remaining. These data are in good agreement with previous results.[19-21] The cell survival could be drawn with a linear quadratic curve ($S = e^{-0.2D-0.095D^2}$).

Surviving Fraction in the Irradiation Field and the Peripheral Zone

The surviving fraction was established for different irradiation doses. Considering the data obtained with the BioBeam irradiator, we chose to irradiate with 2 Gy (50% survival), with the lethal dose (10 Gy) and with a very high dose of 20 Gy. The results are shown in Figure 4. The surviving fraction was 52% ± 5% with 2 Gy irradiation, not significantly different from the value obtained with the BioBeam irradiator. At 10 and 20 Gy, the surviving fraction was very low, about 6%, but was significantly higher than the value obtained at 10 Gy with the whole flask irradiation using the BioBeam irradiator ($P < .001$). We noted that, even with high doses, some cells remained alive in the irradiation field and were able to proliferate. In the peripheral zone, the surviving fractions were significantly lower than in controls for the 3 doses ($P < .01$) and were significantly lower at 10 Gy at the isocenter ($P < .01$) than with 2 Gy irradiation.

Surviving Fraction Per Isodose in the Dose Gradient Zone

We have investigated the cell survival using a clonogenic assay, in the dose gradient zone, according to the different isodoses for doses of 2, 10, and 20 Gy delivered at the isocenter. The results are shown in Figure 5. It can be observed that, as expected, survival increased with decreasing dose. However, higher survival was observed for very low doses at the periphery of the irradiation field, whatever the dose delivered at the isocenter. This trend toward protective effects was statistically significant with a threshold at 0.2 Gy for the 20 Gy isocenter dose ($P < .01$) and at 0.1 Gy for the 10 and 2 Gy isocenter doses ($P < .01$).

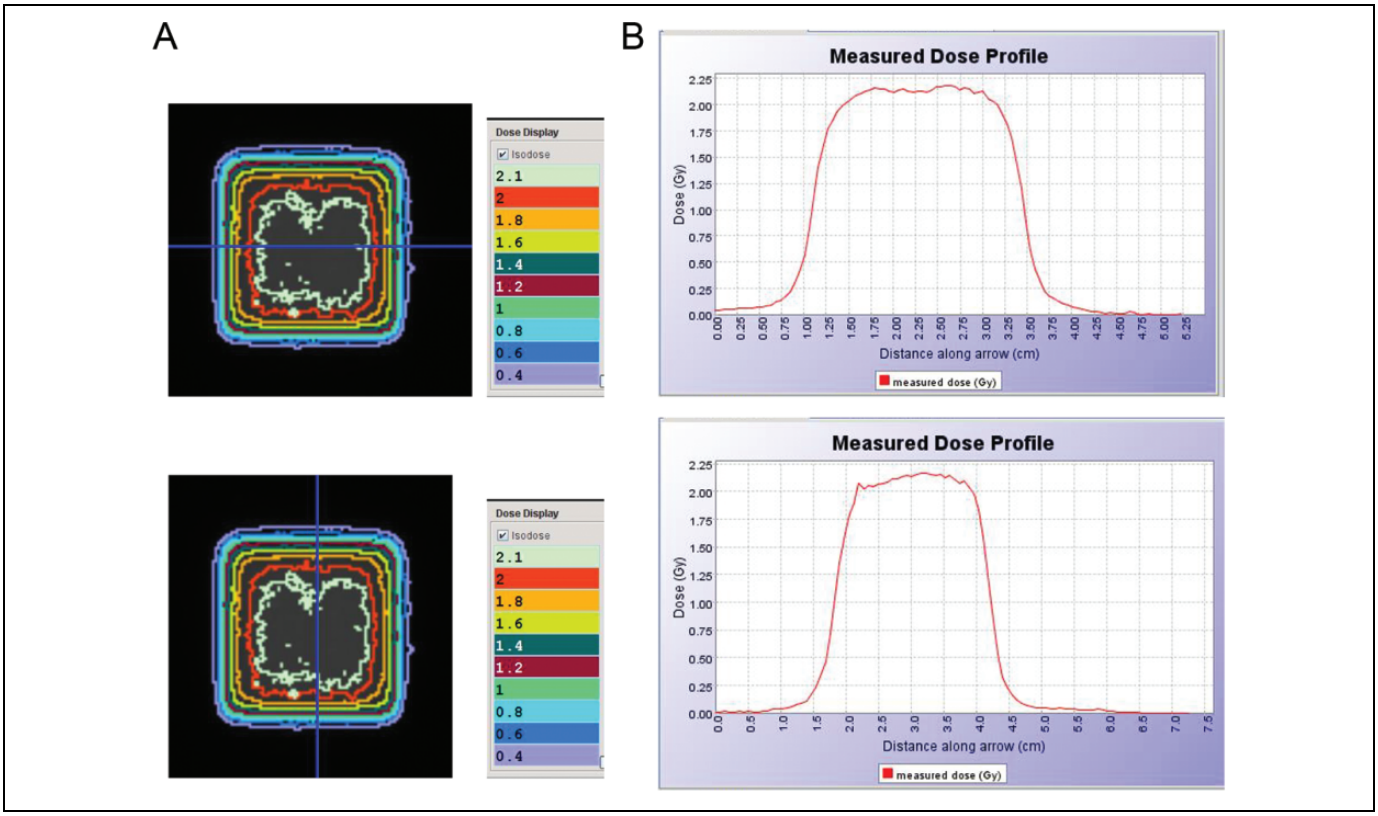

Figure 2. Film dosimetry at the bottom surface of a T25 flask for irradiation with 196 MU. A, Dose distribution. B, Characteristic dose profile along the blue line indicated in panel A.

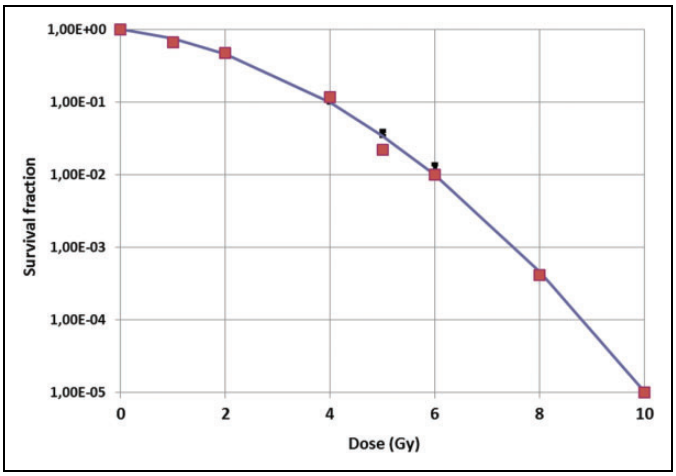

Figure 3. Survival curve of MCF7 cells using the BioBeam irradiator determined by clonogenic survival assay. Each point represents the mean of at least 3 experiments. Cell survival corresponds to a logarithmic curve fitted to the LQ model with $S = e^{-0.2D-0.095D^2}$. Error bars indicate $\pm$ standard error of the mean.

In the peripheral zone considered as unexposed (<0.2 Gy for the isocenter dose of 20 Gy and <0.1 Gy for the isocenter doses of 10 Gy and 2 Gy), the surviving fraction decreased compared to controls, suggesting a loss of the protective effect.

Discussion

With the development of new radiation therapy techniques and particularly IMRT, it is important to characterize cellular response to spatially modulated radiation fields. Survival data with different radiation doses of cultured cells may differ according to whether the whole surface of the flask is irradiated (as with the BioBeam irradiator), or only a limited area of the flask receives most of the radiation dose. The latter condition better reflects the in vivo irradiation of limited tumors where neighboring cells are present.

We chose to use clonogenic assay experiments rather than MTT tests in our study. The MTT experiments consider cell metabolism and are not directly related to cell survival, although they have the advantage of underlining in situ differences in metabolic cellular responses.[12,13]

The cell survival of MCF7 cells irradiated with the BioBeam irradiator was measured in order to confirm the LD50 of MCF7 cells. It was in accordance with data presented in the literature.[19-21] We observed a LD50 at 2 Gy and a lethal dose at 10 Gy, with about 1% of the cells remaining viable at 6 Gy and none at 10 Gy.

We have shown in this study that the cell response to radiation is not the same when neighboring cells are irradiated with different doses. Moreover, when they shared the

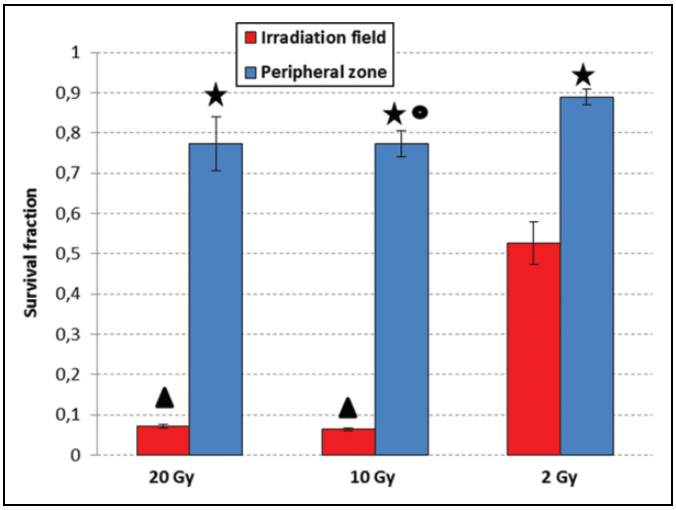

Figure 4. Surviving fractions of MCF7 cells obtained by clonogenic assays for different doses (Varian Novalis irradiator) inside the irradiation field (red) and in the peripheral zone (blue) as compared with nonirradiated cells (control flasks). Error bars indicate ± standard error of the mean. ★ Statistically lower when compared to controls ($P < .01$). ▲ Statistically higher when compared to 10 Gy whole flask irradiation ($P < .01$). ● Statistically lower when compared to 2 Gy peripheral zone ($P < .01$).

same flask as irradiated cells, nonirradiated cells showed lower survival compared to sham irradiated controls. This is the first time that cell survival has been determined in the dose gradient zone for different isodoses and various doses at the isocenter.

Using the linear accelerator, cell survival inside the irradiated field was about 53% at 2 Gy, close to the DL50 obtained with total flask irradiation. However, we did not observe killing of all the cells with 10 or 20 Gy inside the irradiation field (survival of 6% with Varian Novalis vs 0% with BioBeam). Different explanations could be suggested for this discrepancy. First, the energy and the nature of the source of radiation were different (γ-rays vs X-rays) but the relative biological effectiveness (RBE) was about the same.[22] Second, the irradiation time for the delivery of 10 Gy differed between experiments but was nevertheless very close (3.5 minutes with the accelerator and 3.2 minutes with the Biobeam) so the dose rate was approximately the same. This suggests that some cells were able to survive a high radiation dose when neighboring nonirradiated cells were present in the same flask. Some authors have reported this type of bystander effect (type 3) on melanoma and lung cancer cell lines.[12,14,15] Interestingly, the cell survival of nonirradiated cells in the vicinity (peripheral zone) of irradiated cells in the same flask was lower than in controls (classical bystander effect). This decrease was correlated with the radiation dose at the isocenter. This observation has also been described in out-of-field cells with respect to a 6 MV modulated photon beam radiation field.[17,18] In the latter studies, the out-of-field area corresponds to our peripheral zone. Not only cell survival but also DNA damage and repair were modified by this

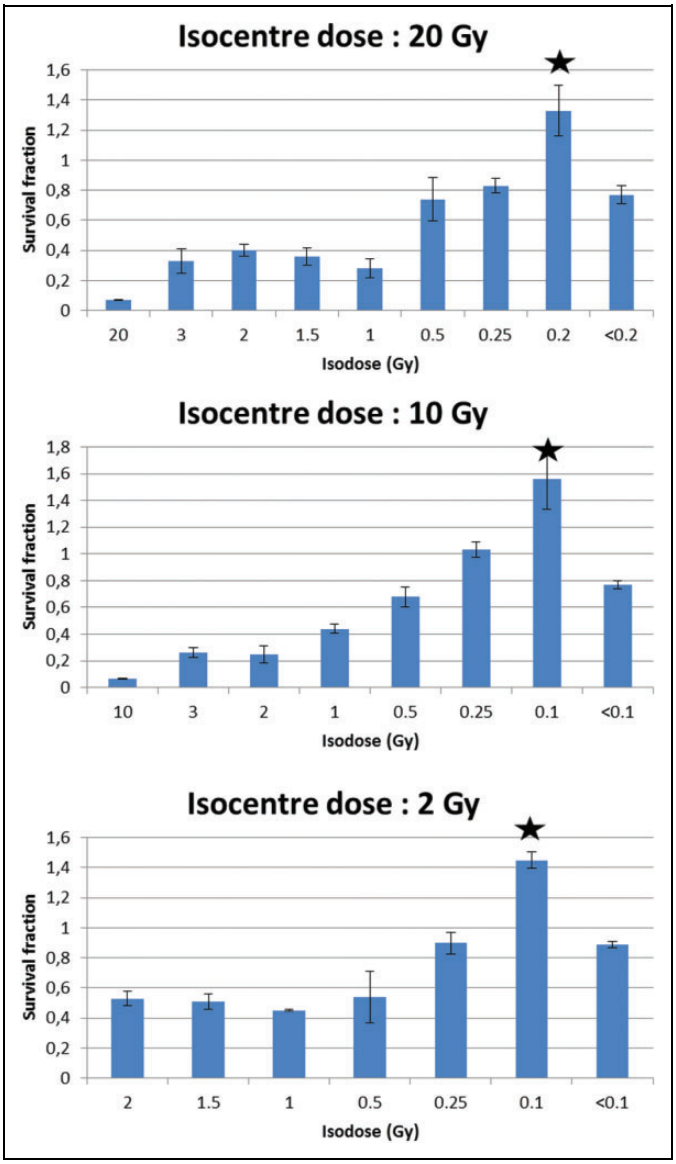

Figure 5. Surviving fractions of MCF7 cells in the dose gradient zone for the different isodoses according to the dose at the isocenter (20, 10, and 2 Gy). ★ Statistically higher compared to peripheral zone or controls ($P < .01$).

bystander effect, as attested by the number of γ-H2AX foci.[23] Our experimental conditions allowed communication to be maintained between cells in the same flask. Unlike other authors, we did not change the medium after irradiation.[12,14,15] Any molecule secreted immediately after irradiation was allowed to diffuse in the medium and act on neighboring cells. Bystander effects cannot be explained by direct communication between cells via gap-junctions since, in the clonogenic assay experiments, the cells were not close to each other, especially immediately after irradiation. So, secreted molecules such as reactive oxygen species or nitric oxide may have been predominant in mediating this effect.[17,23]

In the dose gradient zone, we observed increased cell survival with decreasing irradiation. Interestingly, higher cell

survival was observed at the periphery of the dose gradient zone than in the peripheral zone or the control flasks. This observation may be of special interest when considering radiotherapy schemes. Nonlinear effects of very low doses, such as hyper-radiosensitivity followed by increased radio-resistance, have been described in many cell lines. Hyperradiosensitivity is described for doses of about 0.2 Gy and increased radio-resistance for doses of about 0.5 Gy.[6] Using the BioBeam irradiator, we did not observe such increased survival or hyper-radiosensitivity for doses between 0.05 and 0.25 Gy (data not shown). However, the MTT assay has previously underlined increased metabolic activity compared to controls for doses of about 0.01 Gy at the periphery of the radiation field.[13] In the study presented here, for the same dose level, a clonogenic assay showed increased cell survival.

These observations may have a clinical impact for radiation therapists. They suggest that increased survival of cancer cells may be induced by low-dose irradiation. Thus, the target volume for irradiation may be drawn in such a way that the margins of the irradiated field are located in healthy tissue in order to take in all cancer cells. This is supported by the potential protective effects on normal tissue that we observed in the dose gradient. Similar effects should be explored on normal cells.

Conclusion

In conclusion, using a cell culture model that allowed cell–cell communication via the culture medium, we observed an absence of linearity in the response of irradiated cells when surrounding cells were present, and the medium was unchanged. These different bystander effects should be kept in mind when determining the target volume for irradiation schemes. The delivery of low doses must be controlled to avoid not only the emergence of a second cancer in the surrounding tissue but also radioresistance in the tumoral area.

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Declaration of Conflicting Interests

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