Radiobiological Investigation of Dose-Rate Effects in Intensity-Modulated Radiation Therapy

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Background and Purpose: Intensity-modulated radiation therapy (IMRT) has proven extraordinary capability in physical terms such as target conformity, dose escalation in the target volume, and sparing of neighboring organs at risk. The radiobiological consequences of the protracted dose delivery for cell survival and cell cycle progression are still unclear and shall be examined in this study.
Material and Methods: Human lymphoblasts (TK6) and human melanoma cells (MeWo) were irradiated with protocols of increasing dose protraction. In addition, a new biophysical phantom was developed and used to transfer clinical IMRT plans to experimental cell irradiation. Clonogenic cell survival and cell cycle analysis were performed after various irradiation experiments.
Results: In a first series of experiments, melanoma cells showed a highly significant increase of survival of 6.0% after protracted dose delivery of 2 Gy compared to conventional fast application with the same dose. Lymphoblastoid cells also showed a significant increase of survival of 2.2%. Experiments with patient plans in the phantom confirmed the trend of increased cell survival after protracted dose delivery. Cells were irradiated at 13 points in four different IMRT plans. In comparison to irradiation with application of the same dose in a classic four-field box, a significantly increased survival of 5.1% (mean value) was determined.
Conclusion: Even at fraction times of 15–30 min the protracted dose delivery increases the survival rates in cell culture. The altered survival rates indicate the importance of the dose rate in the effectivity of IMRT. Besides physical parameters the consideration of biological factors might contribute to the optimization of IMRT in the future.

Key Words: IMRT · SMLC IMRT · Step and shoot · Dose rate · Dose protraction · Cell survival

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Strahlenbiologische Untersuchung von Protrahierungseffekten in der intensitätsmodulierten Strahlentherapie

Hintergrund und Ziel: Die intensitätsmodulierte Strahlentherapie (IMRT) ist ein modernes Radiotherapieverfahren, welches unter physikalischen Gesichtspunkten wie der Zielkonformität, Dosiseskalation und Schonung von Risikostrukturen hervorragende Ergebnisse erzielen kann. Doch die strahlenbiologischen Konsequenzen für Zellüberleben und Zellzyklusprogression, die sich aus der protrahierten Dosisapplikation ergeben könnten, sind noch unklar und sollen in dieser Arbeit untersucht werden.
Material und Methodik: Humane Lymphoblasten (TK6) und humane Melanomzellen (MeWo) wurden mit Protokollen ansteigender Dosisprotrahierung bestrahlt. Zudem wurde ein neuartiges biophysikalisches Phantom entwickelt, welches die Übertragung klinischer IMRT-Pläne in ein vielseitiges experimentelles Setup ermöglicht. Klonogenes Zellüberleben sowie Zellzyklusprogression nach verschiedenen Bestrahlungsexperimenten wurden untersucht.
Ergebnisse: In einer ersten Versuchsreihe zeigten die Melanomzellen ein signifikant um 6,0% erhöhtes Zellüberleben, wenn 2 Gy stark protrahiert appliziert wurden, verglichen mit schneller herkömmlicher Bestrahlung. Auch die Lymphoblasten zeigten ein um 2,2% signifikant erhöhtes Überleben. Die Experimente im Phantom mit Patientenplänen bestätigten den Trend des erhöhten Überlebens nach Dosisprotrahierung. Die Zellen wurden an 13 verschiedenen Punkten in vier IMRT-Plänen bestrahlt. Im Vergleich zur Bestrahlung mit der gleichen Dosis in einer konventionellen Vierfelderbox war das Überleben nach IMRT durchschnittlich um 5,1% erhöht.
Schlussfolgerung: Selbst bei Fraktionszeiten von 15–30 min führt die protrahierte Dosisapplikation zu einem erhöhten Zellüberleben in Zellkultur. Die veränderten Überlebensraten zeigen die Bedeutung der Dosisrate für die Effektivität der IMRT. Neben physikalischen Parametern der Planbeurteilung müssen auch biologische Parameter zur weiteren Optimierung der IMRT herangezogen werden.

Schlüsselwörter: IMRT · SMLC-IMRT · Step and shoot · Dosisrate · Dosisprotrahierung · Zellüberleben

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Introduction

After 1 decade of clinical intensity-modulated radiation therapy (IMRT), a lot is known about its capabilities in physical terms such as target conformity, dose escalation in the target volume, and sparing of neighboring organs at risk [6, 10, 16–19, 23, 28]. These qualities permit the irradiation of patients with complex-shaped tumors at problematic locations such as the skull base, which could not be treated successfully with conventional irradiation methods due to tolerance doses of surrounding structures. In addition, very aggressive and radioresistant tumors can be treated with higher doses without increasing the dose to the surrounding tissues.

However, the intensity modulation of the radiation beams requires sophisticated methods which increase the time to deliver one fraction and therefore lower the average dose rate. Different technologies of IMRT are available, of which the step-and-shoot method or segmental multileaf collimator (SMLC) IMRT is widely used. It is performed with a sequence of several subsegments at intervals of several seconds [2]. Complex IMRT plans result in a pulsed dose delivery consisting of > 100 subsegments with a fraction time of up to 30 min. The radiobiological consequences of this decreased dose rate and pulsed dose application are still unclear [8, 18]. Lambin et al. described hypersensitivity reactions after irradiation with low doses up to 0.5 Gy for various cell lines [12]. A sequence of several low-dose pulses might therefore have effects in terms of hypersensitivity and decreased cell survival. Figure 1 shows a hypothetical survival curve of cells displaying cumulative hypersensitivity to low-dose pulses.

Yet, the lowered dose rate might as well allow split-dose recovery during the prolonged time of one fraction and thus diminish the probability of strand break interactions resulting in a higher cell survival [8, 25]. For continuous irradiation at different dose rates an increased survival could be shown by Ruiz de Almodovar et al. [22]. These dose rates are similar 30 min. In addition, enzymes involved in DNA repair could be induced by early hits of radiation and radioresistance would be altered during the radiation fraction [11].

Cell cycle progression during a fraction should be low due to the comparatively long generation times of the cells, but a low percentage of cells might become arrested in resistant cycle phases after early radiation hits and therefore radiation sensitivity might change during the time of a fraction.

To investigate these radiobiological consequences of IMRT, survival rates and cell cycle distributions following irradiations at different dose rates simulating IMRT were examined in cell culture. In addition, a new phantom was developed which enables clinical IMRT plans to be transferred into an experimental setup allowing both physical and biological measurements.

Material and Methods

Cell Culture

Mycoplasm-free human melanoma cells (MeWo) were maintained in monolayer in exponential growth without any antibiotics in RPMI 1640 (Pan) supplemented with 10% fetal calf serum. They were passaged routinely twice a week using a calcium-free salt solution with 0.2% EDTA and 0.2% trypsin (Sigma). The cells are known to have an intermediate intrinsic radiosensitivity showing a surviving fraction after irradiation with 2 Gy of approximately 35% in our pilot experiments.

Mycoplasm-free human lymphoblastoid cells (TK6) were maintained in suspension in RPMI 1640 (Pan) supplemented with 10% heat-inactivated horse serum (Sigma) without addition of antibiotics. Cell concentration ranged between 10^5 and 10^6 cells/ml. Their intrinsic radiosensitivity is very high with a surviving fraction at 2 Gy of approximately 10%.

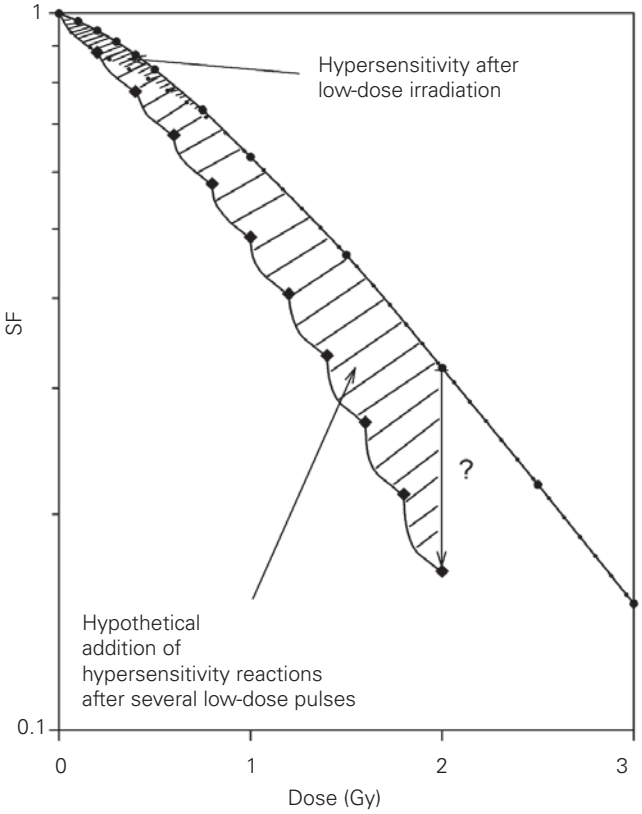


Figure 1. Hypothetical survival curve for added hypersensitivity effects: the black line shows a classic survival curve, the dotted line hypersensitivity after low-dose irradiation, and the gray line the initial part of the dotted curve iterated for several 0.2-Gy pulses resulting in a hypothetical survival curve with decreased survival after 2 Gy.

Abbildung 1. Hypothetische Überlebenskurve für eine Folge von Hypersensitivitätseffekten: Schwarz dargestellt ist die klassische Überlebenskurve, gepunktet die Hypersensitivität im Niedrigdosisbereich und grau die Addition des Anfangsabschnitts der gepunkteten Linie für mehrere 0,2-Gy-Pulse, was hypothetisch zu einem erniedrigten Überleben nach 2 Gy führt.

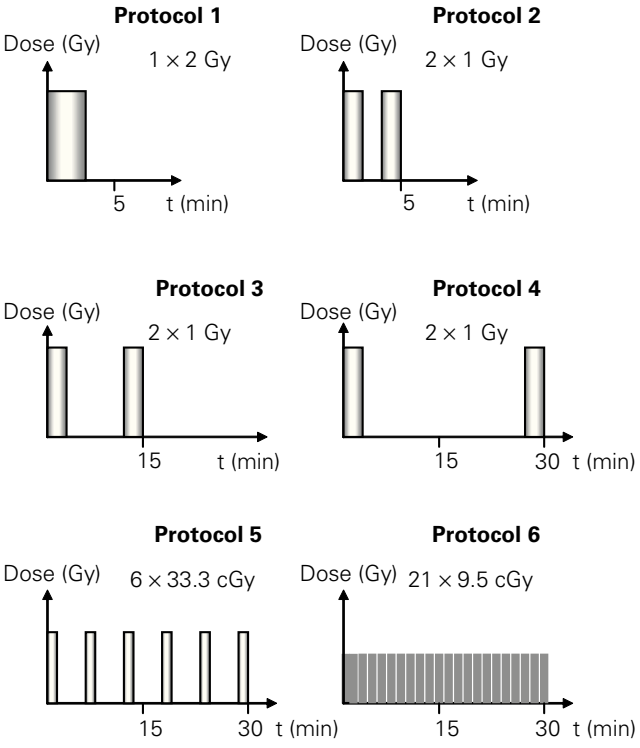


Figure 2. Six irradiation protocols with decreasing dose rate and increasing number of dose pulses, overall dose always 2 Gy.

Abbildung 2. Sechs Bestrahlungsprotokolle mit abnehmender Dosisrate und ansteigender Zahl von Dosispulsen, Gesamtdosis in jedem Plan 2 Gy.

All cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2.

Survival Assays

Clonogenic survival of the adherent melanoma cells was determined using the colony-forming assay. Cells were plated in 25-cm^2 flasks at concentrations producing 100–200 colonies. After irradiation cells were incubated for 14 days and afterwards fixed with 75% methanol/25% acetic acid solution. After staining with 0.1% crystal violet solution colonies with > 50 cells were counted.

Survival of the lymphoblastoid cells in suspension was examined using the microtiter plate dilution assay [7]. 200 µl of cell suspension was pipetted into each of the 96 wells at suitable concentration. Clonogenic survival following Poisson's distribution could be seen after 14 days of incubation by changing of indicator color (phenol red) or through the microscope.

Cell Cycle Analysis

Cell cycle distributions were analyzed using one color flow cytometry. At several times following irradiation cells were washed with phosphate-buffered saline (PBS) and afterwards fixed with 70% ethanol at –20 °C. Cell concentration was approximately 500,000/ml. After an incubation time of 30 min with the alcohol cells were washed twice with PBS and 10 µg RNAse (Sigma) and 900 µl propidiumiodide (Sigma) at a concentration of 20 µg/ml were added. After an incubation time of 30 min cell cycle analysis was performed using a Becton-Dickinson FACScan.

Phantom

A new biophysical phantom of cylindrical shape was developed (Schäfer et al. submitted an article about this phantom, Strahlenther Onkol) using the water-equivalent material PMMA. It contains a revolving and laterally shiftable inner core with nine channels accommodating either ionization chambers or cryotubes for cell experiments. By this arrangement every point of a target volume can be examined in stereotactic coordinates for physical or biological studies after transferring IMRT plans to the phantom.

Irradiations

All irradiations were performed at the Clinical Cooperation Unit Radiotherapy of the German Cancer Research Center (dkfz) in Heidelberg, Germany, using a Siemens linear accelerator PRIMUS at energies of 6 or 15 MeV.

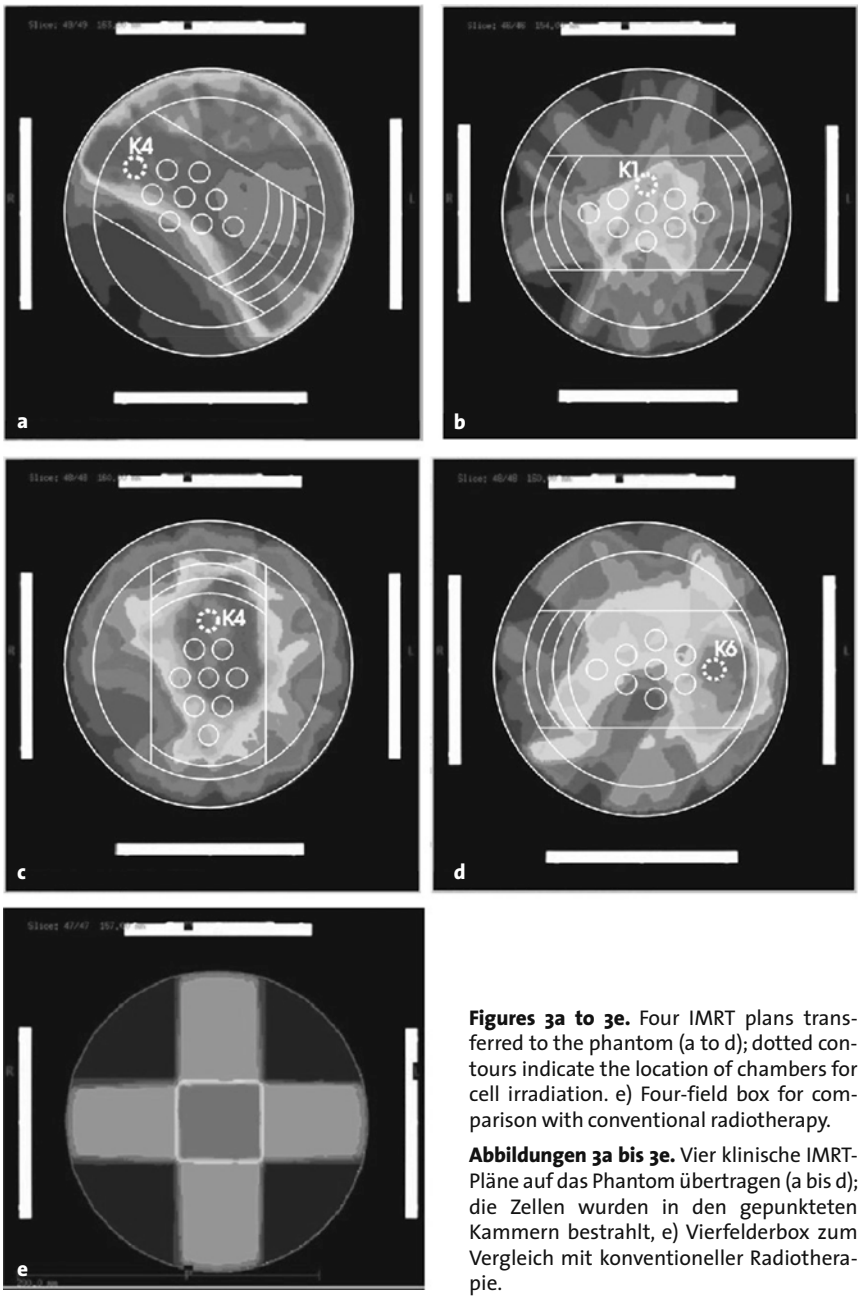
In the preliminary experiments cells were irradiated in 25-cm^2 culture flasks and 96 multiwell plates under RW3 plates 3 cm thick (PTW, Freiburg, Germany). Six different irradiation schedules incorporating a successive decrease of dose rate and a pulsed dose delivery, thus simulating the situation in IMRT, were used (Figure 2). In each protocol a total dose of 2 Gy was applied, at first in one single pulse, then in two pulses with intervals of 5, 15, and 30 min. In the last two protocols the dose was delivered in six and 21 pulses, respectively, each in an overall time of 30 min. The clonogenic survival of the melanoma cells (MeWo) and the lymphoblasts (TK6) was determined as described above.

In the experiments using patient plans, cells were irradiated in cryotubes in the phantom. After trypsinization they were transferred to the cryotubes at suitable concentration and centrifuged to concentrate the cells at the half-ball-shaped bottom of the cryotube. After irradiation cells were resuspended and seeded into 25-cm^2 culture flasks for colony-forming assay.

Four clinical IMRT plans were transferred to the phantom reproducing one case of a sphenoidal meningioma, a nasopharyngeal carcinoma, a carcinoma in the maxillary sinus, and a carcinoma of the breast. These plans were created and transferred to the phantom using the dkfz planning software KONRAD® and VIRTUOS®. Irradiation was performed with the IMRT tool SIMTEC/IMMaxx® [21]. Due to differences between phantom structure and patients' anatomy there were changes in dose distribution. These changes were quite small and, most importantly, the characteristics in temporal dose application determined by number and intensity of subfields remained the same. Figure 3 shows these four plans plus the

four-field box for comparison with conventional radiation therapy; chambers of interest are drawn with dotted contour. Fraction time of the four plans ranged between 15 and 22 min. The cells were irradiated at 13 points of the four plans at doses ranging from 0.59 to 2.47 Gy; corresponding to each point cells were irradiated in the four-field box with the same dose. These points were chosen to gain data at a wide dose range, but a special emphasis was put around 2 Gy representing the most important dose value. Sharp dose gradients were avoided to minimize problems of incorrect positioning of cells. The points were localized at the center of the target volume, at the center of an organ at risk or even very close to such structures to get points with heterogeneous temporal dose distributions.

For dosimetry pinpoint ionization chambers and UNIDOS® and MULTIDOS® dosimeters by PTW, Freiburg, Germany, were used. A new phantom-related software called GRAYHOUND (Schäfer et al. submitted an article about the phantom and this software, Strahlenther Onkol) was used to characterize the temporal dose distribution and dose protraction.



Figures 3a to 3e. Four IMRT plans transferred to the phantom (a to d); dotted contours indicate the location of chambers for cell irradiation. e) Four-field box for comparison with conventional radiotherapy.

Abbildungen 3a bis 3e. Vier klinische IMRT-Pläne auf das Phantom übertragen (a bis d); die Zellen wurden in den gepunkteten Kammern bestrahlt, e) Vierfelderbox zum Vergleich mit konventioneller Radiotherapie.

Results

Preliminary Experiments

Figure 4 shows the average survival data after twelve repeats of the experiments with corresponding standard deviations for both cell lines and the six different irradiation protocols. With an increasing number of pulses and increasing fraction time both cell lines showed elevated survival rates. Especially interesting is the difference between protocol 2 representing a conventional two-field plan and protocol 6 being closest to the protracted dose application in IMRT. Melanoma cells showed a highly significant increase of survival of 7.03% ($p = 0.0002$, t-test) and lymphoblasts an increase of survival of 2.24% ($p = 0.022$, t-test). To illustrate this increase of survival, Figure 5 shows the relative survival data. The ratio of survival fractions clearly indicates that the relative survival increases by factors of up to 1.24. In this calculation the increase of survival of the TK6 cells is more impressive than in absolute values.

Cell cycle distributions at various times after irradiation using protocol 2 and 6 showed no significant differences. Distributions before, immediately after, and 0.5, 1, 2, 4, 6, 8, 16, 24, 48, 72, 96 h after irradiation were analyzed and were almost the same for the two protocols and two cell lines. Both of them showed a clear G2/M-arrest beginning after 2–4 h and reaching a maximum after 16–24 h. Differences between the two protocols did not exceed 1.5% at various points after irradiation and showed no tendency for one treatment schedule.

Experiments Using Patient Plans

Mean survival and standard deviations of ten experiments are presented in Figure 6. Survival curves were fitted with the lin-

ear-quadratic model. Parameters for the melanoma cells and IMRT survival were a = –0.006 ± 0.197 and b = 0.140 ± 0.065, for conventional irradiation a = 0.046 ± 0.016 and b = 0.135 ± 0.052. Differences of survival rates ranged from 2.5% to 10.5%; at seven points the difference was statistically significant after Student's t-test ($p < 0.0001$ to $p = 0.007$). The other six points showed no significant differences with p-values between 0.09 and 0.34.

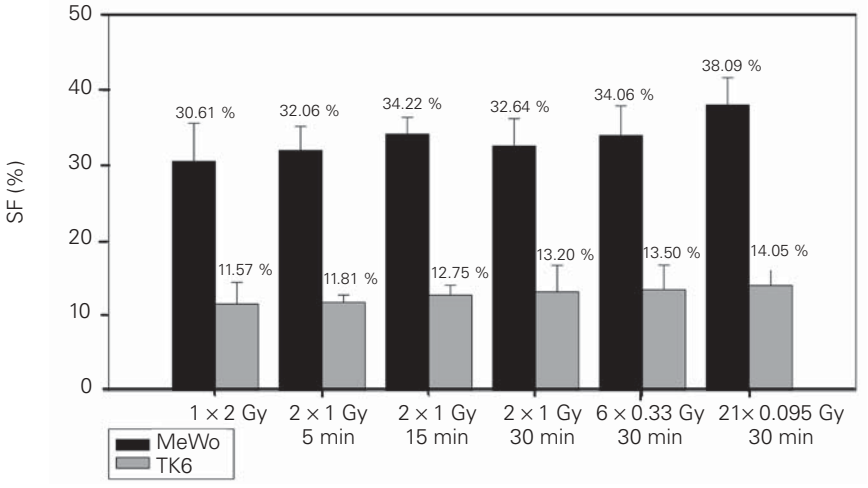


Figure 4. Average survival data and corresponding standard deviations for the two cell lines and six irradiation protocols (twelve repeats).

Abbildung 4. Mittleres Überleben und korrespondierende Standardabweichungen für die beiden Zelllinien und sechs Bestrahlungsprotokolle (zwölfmalige Wiederholung).

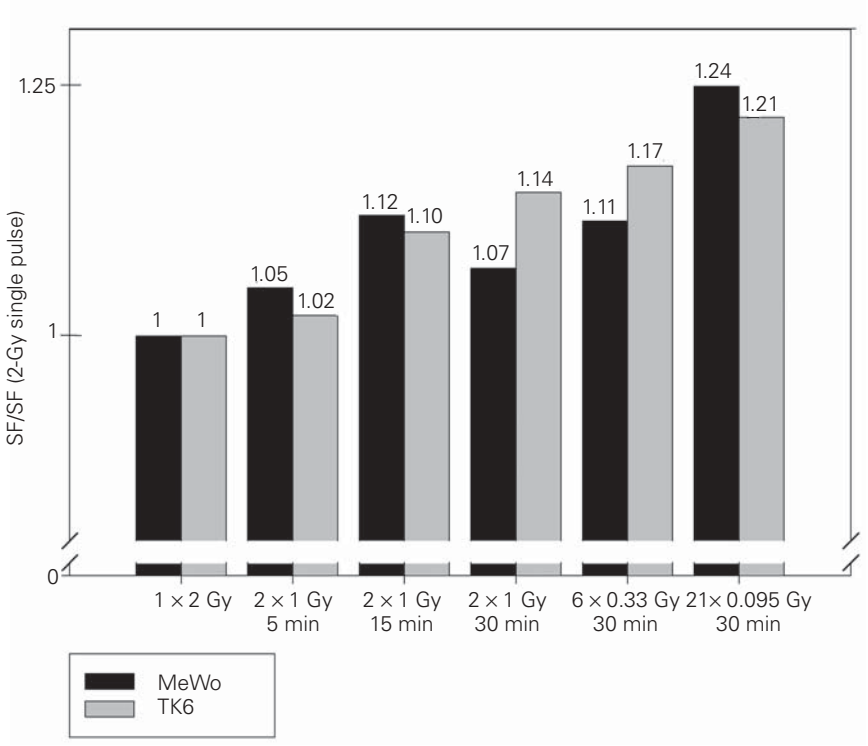


Figure 5. Relative survival data of the two cell lines and six irradiation protocols, normalized to the surviving fraction after 2 Gy delivered acutely (twelve repeats). The ratio of survival fraction and the survival fraction after delivery of 2 Gy in one single pulse of several seconds is shown.

Abbildung 5. Relatives Zellüberleben der beiden Zelllinien für die sechs Bestrahlungsprotokolle (zwölfmalige Wiederholung). Dargestellt ist der Quotient der Überlebensfraktion eines Protokolls und der Überlebensfraktion nach Bestrahlung in einem einzelnen Puls.

Discussion

There are several reports about the excellent therapeutic effectiveness of IMRT in the treatment of prostatic carcinoma and head and neck tumors [7, 26, 27]. Although there is an enormous amount of articles about IMRT, its advantages and new qualities, there are hardly any investigations and considerations regarding biological consequences of the new temporal dose characteristics. A hypersensitive reaction to low doses is known for several cells, so the effect of a sequence of low-dose pulses is difficult to predict. In addition, the speed and capacity of DNA repair in cells make a certain amount of repair during a radiation fraction possible. Stewart & Traub estimated that protracted dose delivery might alter the iso-effect treatment dose at the order of 5–10% [25]. The consequences of protracted dose application have been investigated by Morgan et al., who found increased cell survival after several fractions of protracted dose delivery [14]. Mu et al. tried to analyze this question with different irradiation protocols of increasing fraction time using Chinese hamster fibroblasts V79 for survival experiments. They found a cell survival after protracted dose delivery, that was more increased than biological models predicted [15].

Our results of experiments simulating IMRT characteristics and of experiments using IMRT patient plans made clear that the lowered dose rate has an influence upon cell survival even in a fraction time of 15 min. Dose protraction in patient plans produced an increased survival of melanoma cells in cell culture of 5.1% on average. The MeWo melanoma cells showed an a/b ratio of 0.34 indicating a high capacity of sublethal damage recovery. This may not be typical of all tumor cells, but the results can be seen as a proof of principle.

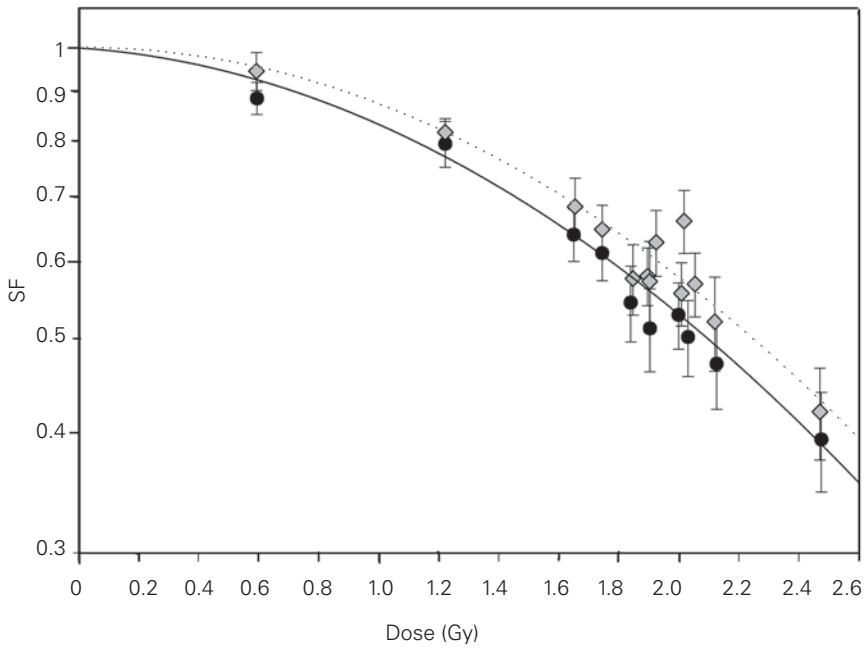


Figure 6. Survival curves of MeWo melanoma cells after irradiation with the four different IMRT plans (dotted line and diamonds) and after irradiation in the four-field box (black line and circles); mean values and standard deviations after ten repetitions.

Abbildung 6. Überlebenskurve der MeWo-Melanomzellen nach Bestrahlung mit den vier verschiedenen IMRT-Plänen (gepunktet und Rautensymbole) und nach Bestrahlung in der Vierfelderbox (schwarz und Kreissymbole); Mittelwerte und Standardabweichungen nach zehn Wiederholungen.

Could these results be a consequence of a systemic error in the experimental setup and methods? In both series of experiments the samples of the different protocols, plans and controls were treated absolutely the same way, beginning from trypsinization, preparation in culture flasks, time out of the incubator up to the counting of colonies, which was performed blindly to prevent any bias influences. Survival rates in the preliminary experiments and in the IMRT experiments were different due to the changes in cell preparation and handling before, during and after irradiation. In the first series of experiments cells were prepared in culture flasks 4 h before irradiation. Afterwards, no more manipulation was done. In the second series cells were filled in cryotubes, centrifuged, irradiated and afterwards resuspended and filled in culture flasks. The irradiation of cell pellets may not be ideal, but this allowed the experiments in a minimal volume and therefore best achievable dose precision. This treatment decreased the plating efficiency from about 40% to approximately 15%. Although the principle of the colony-forming assay remains the same, the experimental setup has to be viewed as a different testing system. Absolute values of survival rates between the preliminary experiments and the IMRT experiments can only carefully be compared.

The precision of positioning cells in the phantom was as high as possible. The laser positioning system of the linear accelerator and a millimeter scaling of the filling cylinders allowed an accuracy of 1 mm. Before irradiation cells were centrifuged to concentrate the cells in an aggregate at the bottom of the half-ball-shaped cryotube. The cells remained in this position even after horizontal placement, so the accuracy here should also be around 1 mm. The overall positioning error should be at most ± 2 mm. Could this positioning error have influence on the measured survival rates? In a plan with inhomogeneous dose distribution as typically present in IMRT, the cells could get into areas of the plan with different doses than expected or previously measured. But there could be either more or less dose than predicted. As long as big dose gradients are avoided and experiments are performed in an intermediate dose level, the probabilities of higher or lower doses should keep a balance. With one exception of the high dose point of 2.47 Gy the examined areas were in median dose level and well away from sharp dose gradients. Dosimetry with pinpoint ionization chambers was done several times befor cell experiments producing an error of measurements of 2%. The mean value of total dose was used for further analyzation of the survival data.

So what does this increased cell survival mean for the effectiveness of IMRT? Does IMRT kill less tumor cells than conventional therapy does? Does the protracted dose delivery diminish advantages such as dose escalation? Could this be a disadvantage of IMRT technologies with a long fraction time like the step-and-shoot technology and should faster technologies be preferred? In that case methods that improve the efficiency of segmentation algorithms or segment delivery would gain importance [1, 3–5, 13, 24]. With long dose protraction dose escalation would not only be a possibility provided by IMRT but rather become a necessity to achieve sufficient cell killing. To calculate the necessary escalation, a parameter like a biological effect equivalent might be useful. Depending on the fraction time and a parameter of radioresistance and DNA repair capacity of different cells, the needed iso-effect treatment dose could be adjusted to the protraction effects.

On the other hand, the increased survival could occur especially in cells with high repair capacity. Normal body cells are known to be more efficient in DNA damage repair than tumor cells. This could mean that especially healthy cells would benefit from the lowered dose rate and tumor cells could not take this advantage that well. The consequence would be a lowered toxicity of radiation to surrounding tis-

sues, something like a "superfractionation during the fraction". There could also be a difference in the reaction of different tumors. The effectiveness of IMRT might be lower in the treatment of tumors with high DNA repair capacity.

There remains a lot of uncertainty in the evaluation and judgment of the presented data. The presented effects were seen in cell culture. Can these results be transferred to the real situation of cells in a tumor with all special parameters such as intercellular interactions or inhomogeneities in blood or oxygen supply? To perform the experiments described in this paper, we had to make a lot of compromises in cell handling and testing procedures. This was necessary to be able to work in the phantom, to use patient IMRT plans, to have the best positioning accuracy, and to be the closest to "IMRT reality" as possible. The presented data must be seen as a proof of principle of protraction effects and an impulse for further experiments to explore the special biology of IMRT.

Conclusion

The presented data show that an increase of fraction time of 15–30 min has a significant influence upon cell survival in cell culture. It seems to allow DNA damage repair during one fraction and lower the probability of lesion interaction. Hypersensitivity reactions after low-dose pulses could not be found in our experiments. The fraction time of 30 min seems to be too short to produce effects and differences in cell cycle progression during or after irradiation. For the first time this could be shown in real IMRT patient plans.

Due to the fact of the described influence of dose rate and dose protraction these parameters should be considered in future optimization of IMRT. Physical qualities such as target conformity or dose escalation are not the only determining factors of therapeutic effectiveness in IMRT.

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