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HETEROGENEITY IN THE FRACTIONATION SENSITIVITIES OF HUMAN TUMOR CELL LINES: STUDIES IN A THREE-DIMENSIONAL MODEL SYSTEM

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Purpose: Current concepts to optimize the therapeutic gain of radiotherapy by hyperfractionation assume that human tumors are less sensitive to fractionation than late reacting normal tissues. The aim of this study was to investigate the extent of the intercell line heterogeneity of fractionation sensitivity of a wide variety of human tumor cell lines in a three-dimensional model system under fully oxic conditions using schedules with one to eight fractions. Biological characteristics of the tumors that correlate with fractionation sensitivity should be identified.

Methods and Materials: A total of 21 cell lines from human tumors maintained as multicellular spheroids consisting of 1000–1500 cells were given fractionated irradiation within a total treatment time of maximally 50 h. Complete dose–spheroid control curves were determined for each fractionation scheme. The spheroid control data were adequately described by the linear quadratic model assuming Poisson statistics. In addition, the induction of a G2 block by a fractionated test dose of seven 3 Gy fractions given at 6-h intervals was determined in spheroid cells using flow cytometry of propidium bromide stained cell nuclei.

Results: The fractionation sensitivities of human tumor cells in multicellular spheroids could be characterized by α/β values, ranging from 2.8–37 Gy in dependence on the cell line. The log normally distributed α/β values were positively correlated with the percentage increase in G2/M phase after the fractionated test dose compared to the controls ($r = 0.72$, $p < 0.01$), and were associated with the degree of tumor differentiation ($p = 0.01$, ANOVA F-test). No significant correlation between the log (α/β) values and the surviving fractions at 2 Gy (SF2) or the total doses with 2 Gy per fraction necessary to control 50% of the spheroids (SCD_{50}) was observed. Despite the intercell line variability of the α/β values, the SCD_{50} values of the different cell lines, given with one and eight fractions or one fraction and 2 Gy per fraction, were closely associated (Spearman rank correlation coefficients: $r = 0.89$ or $r = 0.90$, $p < 0.0001$).

Conclusion: Human tumor cell lines showed a marked heterogeneity in the fractionation sensitivity when irradiated as multicellular spheroids and assayed *in situ* using the spheroid control end point. Therefore, the therapeutic gain of altered fractionation also depends on those biological characteristics of each individual tumor that affects its fractionation sensitivity. Parameters that correlate with fractionation sensitivity of the tumor lines in the spheroid system were identified as grade of tumor differentiation and percentage increase in G2/M cells at the end of an eight-fraction schedule.

Fractionation sensitivity, Multicellular spheroids, Radioresponsiveness, G2 block.

INTRODUCTION

Apart from the fractionation sensitivities of the dose-limiting normal tissues, the fractionation sensitivities of the tumors are one of the most important parameters for optimization of the therapeutic ratio of radiotherapy by altered dose fractionation. Experimental data from rodent tumors support the hypothesis that tumors have low fractionation sensitivities similar to acute reacting normal tissues. When assayed *in vivo* under uniform hypoxic or oxic conditions, rodent tumors showed fractionation sensitivities characterized by a median α/β value of 19 Gy (17, 59, 80). The intertumoral heterogeneity was quite large, with α/β values ranging from 0.9 to 56 Gy.

For the determination of the α/β ratios of human tumor cells as a quantitative measure of fractionation sensitivity, two different classes of end points have been used, the *in situ* end points, tumor control, or growth delay, on the

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one hand, and cell survival in the clonogenic assay on the other. Experiments using the *in situ* end points more closely resemble the clinical situation. Interactions between cells after therapy are preserved. However, data on *in situ* measured fractionation sensitivities of human tumor cells in three-dimensional tumor systems, xenografts, or multicellular spheroids, are only available from a few studies (26, 32, 57, 62, 64, 66, 77, 83). Most of the studies carried out up to now have used the colony formation test as an end point to quantify the radiation effect after various fractionation schedules. In this case, the tumor cells were irradiated either as single cells in suspension (21, 42, 59, 64), on plastic substrate (35, 42, 59), as plateau phase monolayers (4, 33, 83), as spheroids (10, 20, 52, 60), or as xenografts (25, 50, 60). The fractionation schedules included single and fractionated high dose rate, as well as low dose-rate irradiations. Only studies in which effects of varying fractionation schedules were compared represent a functional test of fractionation sensitivity and allow for their quantification. The largest set of data on the fractionation sensitivities of human tumor cells from a single institution was published by Peacock *et al.* (42). In this study, 11 tumor cell lines were treated as single cells in the exponential growth phase either with acute single and split dose or with low dose rate irradiation. The mean α_{LDR}/β_{RR} value was 12.5 Gy with little intercell line heterogeneity in that sample of human tumor lines. Further studies are needed to evaluate the extent of intercell line heterogeneity of the fractionation sensitivities of human tumor cells and those biological characteristics that influence their α/β values. Such information should be an important base for a better estimation of the potential gain of altered fractionation in different clinical situations.

The aims of the present study were to estimate the α/β ratios and their intercell line heterogeneity in a large panel of human sarcoma, glioma, and carcinoma cell lines in a three-dimensional model system under oxic conditions. The multicellular spheroid model system was used that allows for the estimation of the fractionation sensitivity in a proliferative state of a three-dimensional cell organization similar to that of tumors *in vivo*. It should be determined whether the fractionation effects on spheroid control probability can be described by the linear quadratic model assuming complete recovery between fractions. For the applicability of the linear-quadratic model for multifraction irradiations, it is important to have a large set of data supporting or showing limitations of this model. Spheroid control was used as an end point providing the necessary resolution to show small differences between fractionation arms. The present report summarizes spheroid control experiments from 21 human tumor cell lines. This set of data permits the study of associations between the α/β ratios as a measure of the fractionation sensitivity on one side, and the radioresponsiveness, the grade of tumor differentiation and kinetic parameters, as well as the spheroid volume doubling times and the accumulation in the G2 phase during fractionated irradiation on the other.

METHODS AND MATERIALS

Cell lines

Four cell lines, ESS2, EAS1, ENF3, and EL7 stemmed from human grade 2 soft tissue sarcomas, the cell line EF8 stemmed from a lung metastasis of an undifferentiated sarcoma, and EPG1 was from a malignant paraganglioma. The 10 cell lines, EA14, EA3, EA7, EO1, HTZ17, EA12, EA9, U373, A7, and U87 originated from human malignant gliomas, and EPF1 was established from a pilocytic astrocytoma grade 1. In addition, the differentiated and undifferentiated breast cancer cell lines MCF-7 and MDA-MB-435S, as well as the squamous carcinoma cell line FaDu and the adenocarcinoma cell line WiDr have been used. All cell lines beginning with E were established from biopsies in this laboratory. All these cell lines with the exception of EPF1 are immortal and except for ESS2, EA14 and EPF1 grow as xenografts in the flanks of nude mice after subcutaneous injection of 10×10^6 cells. Apart from WiDr, the characteristics of the above cell lines have been described previously (62, 64, 65). The line WiDr was obtained commercially,[1] showing hyperploid DNA content, epithelial morphology, and anchorage-independent growth in the spheroid system or as xenografts in nu/nu mice, and expresses the human enzymes glucose-6-phosphate dehydrogenase type B and lactate dehydrogenase H4 through M4.

Spheroid control assay

This assay has been described in detail elsewhere (64, 65). Spheroids were aggregated from 1500 cells of contact inhibited and 1000 cells of anchorage-independent growing cell lines in cell culture medium overlaying the agarose (1% w/v) coated basal surfaces of 24-well plates. The cell culture medium consisted of Eagle's minimal essential medium supplemented with 10% fetal calf serum, nonessential amino acids, penicillin (100 U/ml), and 5% of a serum extender.[2] Compact spheroids were formed 4 to 5 days after initiation of aggregation. The geometric mean diameters of spheroids from the same cell line showed variation coefficients between 3–10%. Spheroids were irradiated with ^{60}Co-γ-rays at a dose rate of 1.8–2.5 Gy/min at 15°C. At this reduced temperature the small spheroids were fully oxygenated. This was screened in

[1] American Type Culture Collection (ATCC), Rockville, MD.

[2] Seru Max, Sigma, Munich, Germany.

separate measurements of the central pO_2 using polarographic microelectrodes in ≥ 4 spheroids from each cell line attached to cell culture plastic cover slips and overlayered with Locke's solution 5 mm in height (63). For each dose group of the different fractionation schedules, 10–12 spheroids from glioma or carcinoma lines and 16–24 spheroids from sarcoma cell lines were irradiated. A total of 5 to 11 dose groups were studied for each fractionation schedule. The time intervals between dose fractions consisted of a 6 h incubation at 37°C under a 5% CO_2/95% air atmosphere and a 15 min controlled cooling to 15°C in modular incubation chambers prior to irradiation. Forty-eight hours after irradiation, spheroids were transferred individually to uncoated 24-well plates where they became attached. A spheroid was scored as controlled if it did not grow out to a confluent monolayer of more than 50,000 cells during a minimum follow-up period of 3 months. If regrowth started in one or more wells of a treatment group during the last 3 weeks of this observation period, then the follow-up period was prolonged for a minimum of 3 weeks or until confluence was reached in each of the wells with regrowing spheroids.

Cell cycle distribution measurements

The distribution of the DNA content in cells from multicellular spheroids was determined by flow cytometry after preparing single cell suspensions that were incubated with RNase and pepsin. Subsequently, cell nuclei were stained with ethidium bromide. The percentages of cells in the G1, S, and G2/M phases were obtained from the DNA content distributions using a curve-fitting procedure (61).

Radiobiological models and statistical methods

For irradiations with one, two, four, or five, and eight fractions, the radiation doses necessary to control 50% of the spheroids (SCD_{50}) were determined from the numbers of controlled and recurrent spheroids of the different dose groups according to the probit method (Procedures Probit, SAS (54)). Direct analysis of the spheroid control data according to the linear quadratic model assuming Poisson statistics has been described elsewhere (64, 73). The dose–response relationships fitted to the spheroid control data are also given in the Appendix.

To check whether the mean radiosensitivity parameter α and, accordingly, the recovery capacity α/β change with increasing numbers n of fractions of the schedules, the spheroid control data from all fractionation schedules of an experiment were also analyzed according to a more complex model in which an α-modifying factor $\delta(n)$ was introduced as described in the Appendix.

RESULTS

Spheroid control curves after fractionated irradiation are given in Figs. 1 and 2 for the glioma lines EA9 and EO1. Both cell lines showed a significant dose fractionation effect that lead to an increase in the SCD_{50} values and their 95% confidence intervals from 11.8 (11.0–12.4) Gy and 14.1 (13.6–14.9) Gy after 1 fraction to 18.9 (16.6–20.9) Gy and 27.8 (26.3–29.6) Gy after eight fractions, respectively, as estimated by Probit analysis. Spheroid control data after fractionated irradiation for a further six glioma lines and the carcinoma line WiDr are given in Table 1. Fractionation sensitivities of an additional three glioma, three carcinoma, and six soft tissue tumor lines previously published have been included in the following studies on the variability of α/β values and for correlation with biological characteristics (62, 64, 65). The spheroid control data for all 21 cell lines were obtained over a period of 3 years by the same technicians, and only four serum batches were used. Therefore, the coefficient of variation for the $\log(\alpha/\beta_P)$ values was 3.4% on average for retest experiments with eight different cell lines over the total period of the study. Apart from the SCD_{50} values, the α/β values are the most stable and unbiased parameters that can be estimated from the spheroid control data after fractionated irradiation using the α/β model and Poisson statistics (62). Overall, the assay conditions remained constant during the period of study. The follow-up periods for spheroid control experiments were only terminated when spheroids no longer recurred for more than 3 weeks in all treatment groups. Follow-up times of 3–4 months are necessary to observe the latest recurring spheroids, as is demonstrated in Fig. 3. This figure shows the SCD_{50} values after eight fractions ($SCD_{50\ 8fract}$) calculated according to the Probit method from the number of those spheroids constituting a confluent monolayer at the corresponding follow-up time. Most of the human tumor lines reached a plateau for SCD_{50} values only after 90 days.

The α/β_P values according to the Poisson model and their 95% confidence intervals were determined according to the Fieller theorem from the α and β values, their variances and covariance (13). The α and β values and their 95% joint confidence ellipses for glioma lines are shown in Fig. 4a, and for sarcoma and carcinoma lines, in Fig. 4b. These ellipses were calculated from the variances of α and β and their covariance obtained by the fit of Eq. 1 to the spheroid control data (46). Considerable intercell line heterogeneity was seen both in the α, as well as the β values with intercell line coefficients of variation of 36 and 54%. The distributions of both parameters are compatible with a normal distribution. Overall, there is a positive correlation between the α and β values for the 21 human tumor cell lines ($r = 0.45$, $p = 0.04$). Using a linear model to describe the relation between α and β, the slope of the regression line of α on β has the value of 2.5 ± 1.1 Gy ($p = 0.043$), but the intercept is significantly positive with a value of 0.20 ± 0.04 Gy^{-1} ($p < 0.0002$). Therefore, α/β cannot be regarded as a

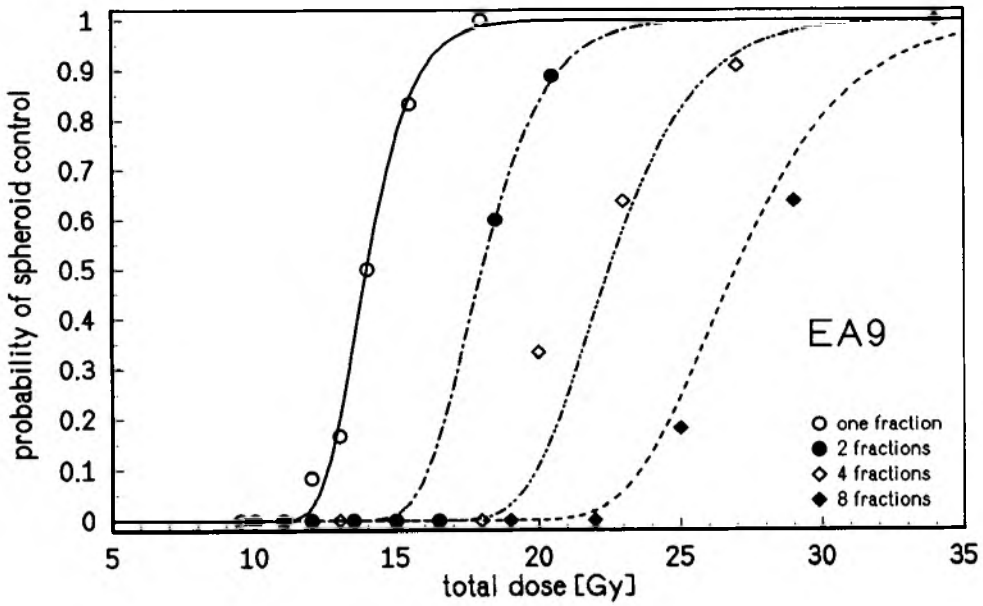

Fig. 1. Control rates for spheroids of the glioma line EA9 after fractionated irradiation. The regression curves are maximum likelihood estimates according to the linear quadratic model assuming Poisson statistics.

constant in this sample of human tumor cell lines, but α/β increases with decreasing α.

The distribution of the α/β values in the population of the human tumor cell lines was compatible with a log-normal distribution [$p = 0.4$, Shapiro-Wilk test (Proc Univariate (SAS)] (54), but not with a normal distribution ($p < 0.001$). The deviations of the $\log(\alpha/\beta_L)$ values according to the logistic model from those obtained from the Poisson model [$\log(\alpha/\beta_P)$] ranged from +24% to −3%, with an average of +2% for the various cell lines. The correlation coefficient between the $\log(\alpha/\beta_L)$ and $\log(\alpha/\beta_P)$ values was $r = 0.99$ ($p < 0.0001$), showing good agreement.

Factors influencing the α/β values

We studied covariations of α/β values from the 21 cell lines with some biological characteristics of the tumor lines. Correlations may be of value for the prediction of the fractionation sensitivities of individual tumors or can help to support or reject concepts as to the mechanisms on which fractionation sensitivity may depend.

In Fig. 5, the $\log(\alpha/\beta_P)$ values were plotted against the calculated SCD_{50} values after irradiation with 2 Gy per fraction ($SCD_{50\,2\,Gy/fract}$). The $SCD_{50\,2\,Gy/fract}$ were calculated from the parameter estimates according to the linear quadratic model assuming Poisson statistics as described elsewhere (65). No significant correlation was found between

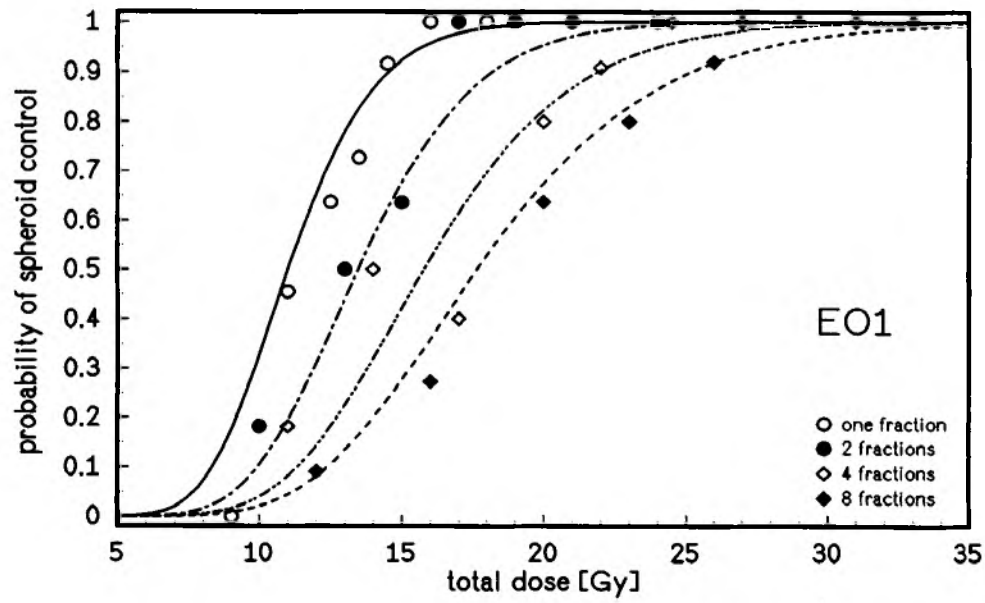

Fig. 2. Spheroid control curves for the glioma line EO1.

Table 1. Spheroid control doses after fractionated irradiation and parameter estimates from the spheroid control data according to the linear quadratic model assuming poisson statistics

Cell-line	1 Fraction SCD_{50} (Gy)	2 fractions SCD_{50} (Gy)	4 Fractions SCD_{50} (Gy)	8 Fractions SCD_{50} (Gy)	α (Gy^{-1})	β (Gy^{-2})	α/β_P (Gy)
EA14	8.7	11.9	14.3	15.9	0.27	0.041	6.7
	(8.2–9.2)	(11.1–13.0)	(13.3–15.3)	(14.1–17.8)	(0.19–0.35)	(0.030–0.052)	(4.8–9.3)
EA3	9.7	12.3	14.9	16.7	0.37	0.044	8.3
	(9.2–10.4)	(11.7–13.3)	(14.0–15.9)	(15.8–17.5)	(0.32–0.43)	(0.038–0.051)	(7.2–9.6)
EA7	10.9	13.8	17.1	18.3	0.28	0.029	9.7
	(10.2–11.5)	(12.7–14.8)	(15.6–18.1)	(16.7–19.7)	(0.22–0.34)	(0.023–0.035)	(7.6–12.3)
EO1	11.8	13.0	15.8	18.9	0.17	0.014	12.4
	(11.0–12.4)	(11.5–14.1)	(13.5–17.6)	(16.6–20.9)	(0.13–0.22)	(0.010–0.018)	(10.8–14.2)
HTZ17	12.1	14.9	20.0	21.8	0.26	0.032	8.3
	(11.6–12.6)	(14.3–15.6)	(18.7–21.5)	(20.5–23.2)	(0.19–0.33)	(0.024–0.039)	(6.5–10.5)
EA12	12.6	16.0	20.3	24.0	0.23	0.032	7.1
	(12.0–13.0)	(15.2–16.7)	(19.4–21.9)	(22.6–26.2)	(0.17–0.29)	(0.026–0.038)	(5.6–8.8)
EA9	14.1	18.6	21.8	27.8	0.20	0.025	8.0
	(13.6–14.9)	(17.8–19.4)	(20.6–23.3)	(26.3–29.6)	(0.16–0.24)	(0.020–0.030)	(6.6–9.7)
U373	14.8	18.2	23.3	23.7	0.24	0.016	15.0
	(14.1–15.6)	(17.3–19.0)	(22.1–24.6)	(22.1–25.3)	(0.19–0.29)	(0.012–0.019)	(11.9–19.2)
WiDr	14.2	19.1	22.2	26.0	0.23	0.022	10.5
	(13.5–14.9)	(18.2–20.1)	(20.9–23.5)	(24.7–27.4)	(0.18–0.27)	(0.018–0.025)	(8.8–12.5)

The parameter estimates for the eight cell lines from malignant gliomas and the adenocarcinoma line WiDr are given together with their 95% confidence limits.

these parameters of radioresponsiveness and fractionation sensitivity in the panel of all 21 cell lines studied (r = 0.27, p = 0.23). The $SCD_{50\ 2\ Gy/fract}$ values only depend on the SF2 values and the number of stem cells k per spheroid. There was also no significant association between the $\log(\alpha/\beta_P)$ and the SF2 values (r = 0.00, p = 1.00). The SF2 values were estimated from the α and β values.

Figure 5 also clearly shows the intercell line heterogeneity of the $\log(\alpha/\beta_P)$ values in the spheroid model. The precision of the α/β_P estimates can be described by the quotient between the upper and lower limit of the 95% confidence intervals, with a mean of 1.8 and ranging from 1.3 to 2.8. This results in standard errors of on average 8% (3–23%) for the $\log(\alpha/\beta_P)$ estimates of the different cell lines. The intercell line coefficient of variation be-

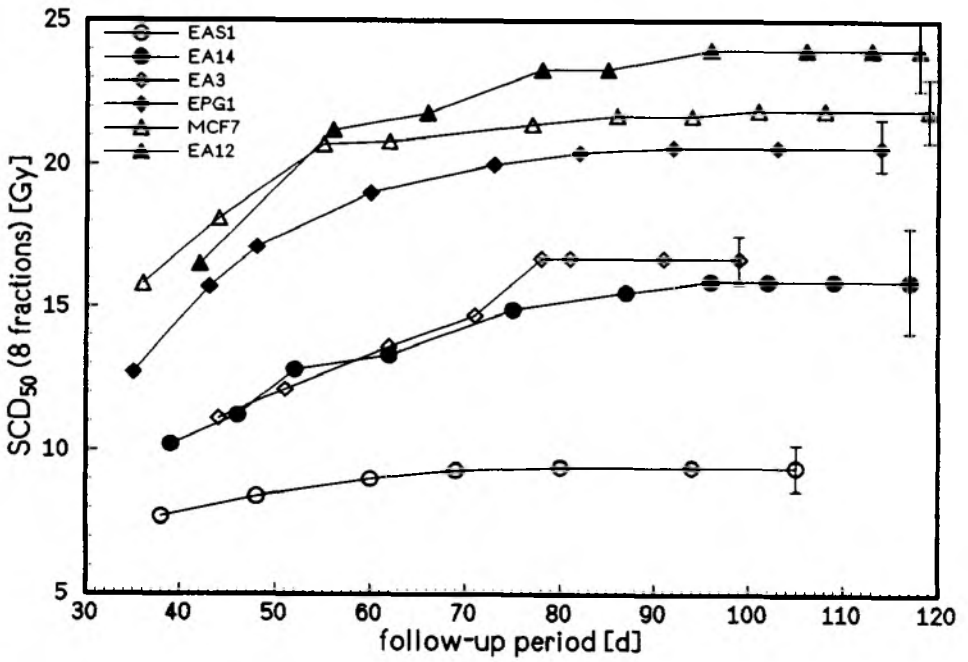

Fig. 3. Increases in radiation doses necessary for a spheroid control probability of 50% (SCD_{50}) given in eight fractions in the course of the follow-up period. The SCD_{50} values are probit estimates from the percentages of spheroids in the different dose groups that had reached the end point at the given follow-up times.

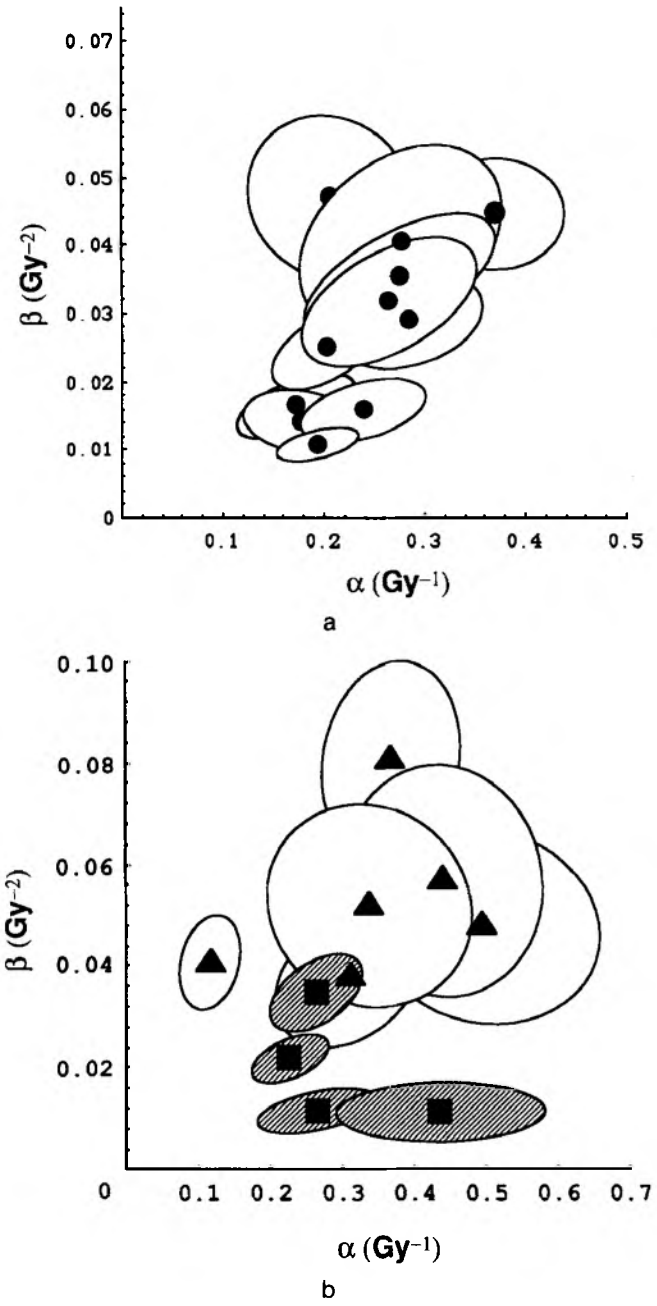

Fig. 4. Scatter plot of the (α,β) data pairs for 11 human gliomas (a) as well as for human (▲) soft tissue tumors and (■) carcinomas (b). The α and β values were estimated from the spheroid control data after fractionated irradiation by the linear quadratic model assuming Poisson statistics and were given together with their 95% joint confidence regions.

tween the log(α/β_P) values for the individual cell lines was 26%. A significant intercell line heterogeneity in the log(α/β_P) values was also found using the method of multiple comparisons of means with the aid of Bonferroni t-tests (38). The 21 cell lines could be partitioned into seven groups with nonsignificant heterogeneity according to their log(α/β_P) values. The smallest group contains α/β_P values ranging from 2.8 to 7.1 Gy, and the largest α/β_P values from 23.3–37.9 Gy.

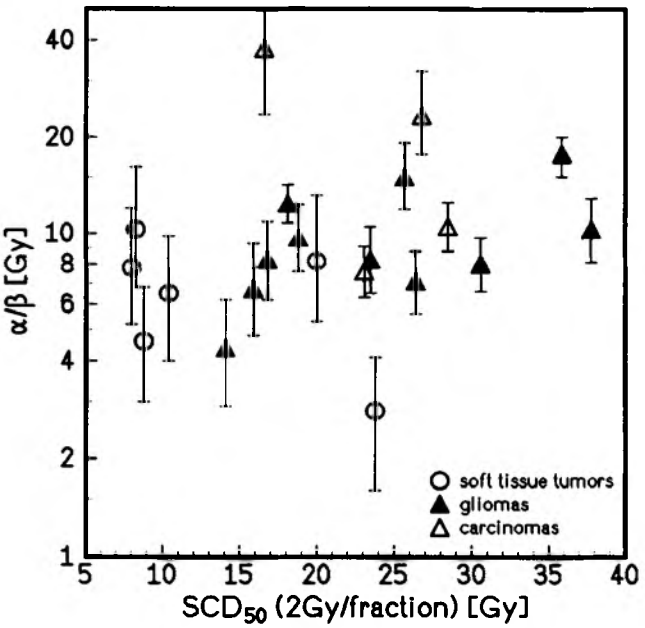

Fig. 5. Scatter plot of the log(α/β) values vs. the calculated SCD_{50} values given with 2 Gy per fraction for 21 human tumor cell lines. α/β values are given together with their 95% confidence intervals. No significant correlation between these parameters of fractionation sensitivity and radioresponsiveness was observed (r = 0.27, p = 0.23).

Figure 6 shows the log(α/β_P) values for the four differentiated soft tissue sarcoma lines ESS2, EAS1, ENF3, and EL7, and the undifferentiated sarcoma line EF8, the differentiated glioma line EPF1, as well as the 10 malignant glioma lines and the differentiated and undifferentiated breast cancer line MCF7 and MDA-MB-435S, respectively, in dependence on tumor type and tumor differentiation. The criteria for the classification as differentiated and undifferentiated have been reported elsewhere (65). Spheroids from the undifferentiated soft tissue sarcoma line EF8 showed a shorter volume dou-

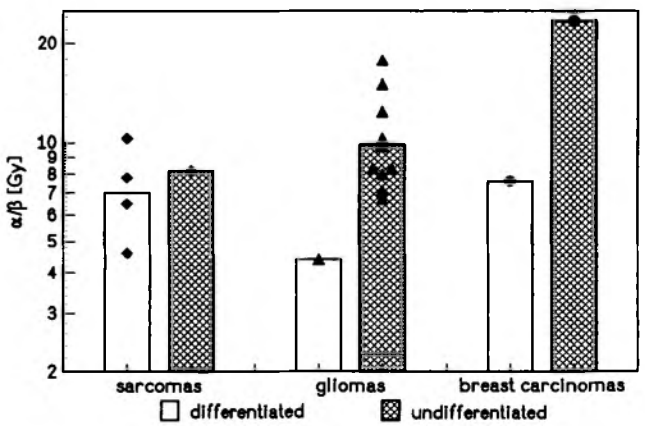

Fig. 6. Classification of the log(α/β) values according to tumor type and grade of tumor differentiation. The influence of tumor differentiation (p = 0.01, ANOVA F-test), but not of tumor type, became significant.

bling time (vdt) of 3.0 days than spheroids from the differentiated sarcoma lines ESS2, EAS1, ENF3, and EL7 (4.3 to > 50 days; median, 5.1 days). The 10 malignant glioma cell lines with volume doubling times between 1.8 and 25.3 days (median, 9.8 days) also showed more rapid growth kinetics in the spheroid model than the cell line EPF1 from an astrocytoma grade 1 (vdt > 50 days). The α/β_P values for the soft tissue sarcomas ranged between 4.6 and 10.3 Gy, those for the gliomas and the mammary carcinomas between 4.4 and 17.8 Gy, or 7.6 and 23.3 Gy, respectively. The corresponding geometric mean α/β_P values for these tumor types were 7.2, 9.2, and 13.3 Gy. In the analysis of variance, only the effect of tumor differentiation on the $\log(\alpha/\beta_P)$ was found to be significant ($p = 0.01$, Type I ANOVA F-test), not the effect of tumor type ($p = 0.13$).

To check whether a correlation exists between the fractionation sensitivity of spheroids from different cell lines and a radiation-induced synchronization through blockage of cell cycle progression during the course of fractionated irradiation, flow cytometric determinations of the DNA content of the cells in spheroids were carried out at different time intervals. The percentage increase of cells in the G2/M phase in multicellular spheroids 6 h after the last of seven 3.0 Gy fractions given at intervals of 6 h compared with unirradiated controls was determined as a measure of radiation-induced cell cycle blockage in the G2 phase in a subgroup of 14 cell lines with differing fractionation sensitivity. The experiments on cell cycle distribution before and after fractionated radiotherapy were repeated twice for each cell line. The culture conditions were identical to those used in spheroid control experiments. The test dose of 7 × 3.0 Gy was chosen in accordance with the mean $SCD_{50\ 8\ fract}$ value for malignant glioma lines, which was 23.4 Gy. The cell cycle distribution measured 6 h after the seventh dose fraction corresponds to that at the end of the eight fraction schedule in the spheroid control experiments. Figure 7 shows the plot of $\log(\alpha/\beta_P)$ vs. the ratio between the percentages of cells in the G2/M phase 6 h after 7 × 3.0 Gy and in the respective control group (percentage increase in G2/M phase). There was a significant positive correlation between the $\log(\alpha/\beta_P)$ values and the parameter for the G2 blockage ($r = 0.72$, $p = 0.004$). The smaller the increase in G2/M phase, the higher the fractionation sensitivity. Other parameters from the DNA histograms, such as the G2/M or S phase content of the controls, did not correlate significantly with the $\log(\alpha/\beta_P)$ values ($r = -0.09$ and $r = 0.22$). In addition, it should be mentioned that the increases in G2/M and the spheroid volume doubling times (vdt) as proliferation dependent parameters were associated with a significantly negative correlation coefficient ($r = -0.64$, $p = 0.01$) and that the $\log(\alpha/\beta_P)$ values from the different cell lines were weakly associated with the log(vdt) values as a measure of the cycling status of the cells ($r = -0,48$, $p = 0.025$).

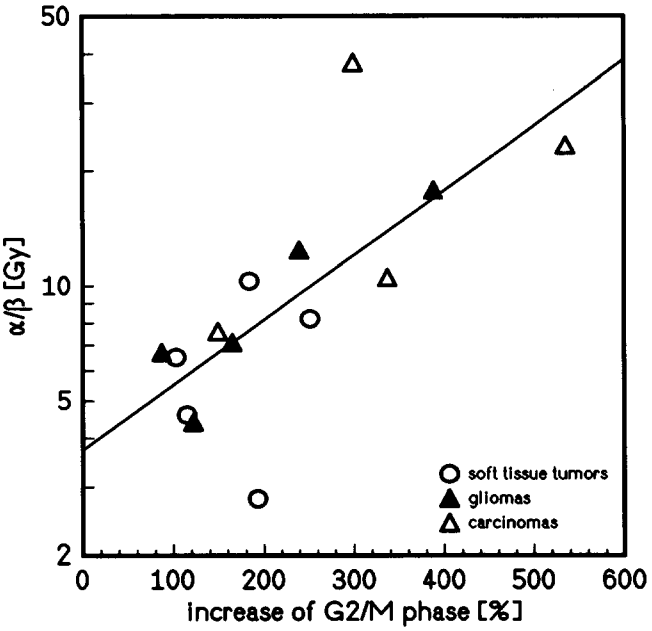

Fig. 7. Correlation between the $\log(\alpha/\beta)$ values and the percentage increases in G2/M phase in a subset of 14 cell lines 6 h after a test dose of 7 × 3 Gy, given at 6 h intervals ($r = 0.72$, $p = 0.004$).

The intercell line heterogeneity of the measured $SCD_{50\ 1\ fract}$ and $SCD_{50\ 8\ fract}$ values is shown in Fig. 8 for the 17 tumor lines for which these data pairs exist. If the α/β values were constant in this sample of human tumor lines, an upward convex relation would hold between the $SCD_{50\ 1\ fract}$ and $SCD_{50\ 8\ fract}$ values according to Eq. 6, given in the Appendix. This relation is shown as a bold dashed curve in Fig. 8. The mean α/β value (12.0 ± 1.5 Gy) was determined according to the least squares method from the $SCD_{50\ 1\ fract}$–$SCD_{50\ 8\ fract}$ data pairs. The 95% confidence region for the prediction of the $SCD_{50\ 8\ fract}$ values from the $SCD_{50\ 1\ fract}$ values is limited by the dotted curves. It shows a width of 13.5 Gy and is an expression of the deviations caused through differences in the fractionation sensitivities of the different tumor lines and also, to a small extent, to random measurement errors. Because the α/β values tend to increase in tumors with larger $SCD_{50\ 1\ fract}$ values, this results in a linearization of the relationship between the $SCD_{50\ 1\ fract}$ and the $SCD_{50\ 8\ fract}$ values. The linear relation according to the regression of $SCD_{50\ 8\ fract}$ on the $SCD_{50\ 1\ fract}$ values is shown as a bold line in Fig. 8. The Pearson coefficient of correlation is $r = 0.93$ ($p < 0.0001$), the Spearman rank correlation coefficient is $r_s = 0.89$ ($p < 0.0001$). In addition, the association between the rankings of all 21 cell lines according to the measured $SCD_{50\ 1\ fract}$ values and the calculated $SCD_{50\ 2\ Gy/fract}$ values was significant ($r_s = 0.90$, $p < 0.0001$).

DISCUSSION

The aggregation of human tumor cells into three-dimensional multicellular spheroids can profoundly alter

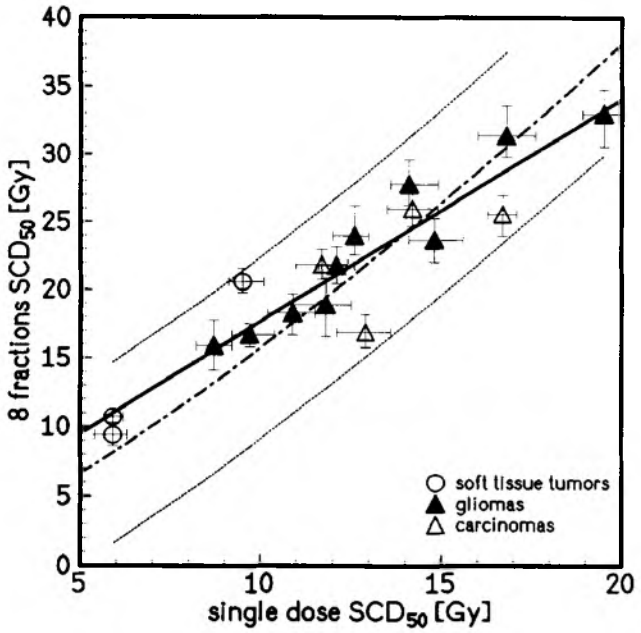

Fig. 8. Scatter plot of the SCD_{50} data pairs for fractionation schedules with one and eight fractions. Data are given together with their 95% confidence intervals. The least squares relation between the single dose and eight fractions SCD_{50} value pairs according to the linear quadratic model assuming complete recovery between fractions is represented by the dashed line. The 95% confidence limits for the prediction of an eight fractions SCD_{50} value from a single dose SCD_{50} value according to the linear quadratic model are given by the dotted curves. The best linear relation between the single dose and eight fractions SCD_{50} values is given by the bold line ($r = 0.93$, $p < 0.001$).

growth regulation (30), expression of proteins, such as extracellular matrix collagens (1), the carcinoembryonic antigen (68), or radiosensitivity and fractionation sensitivity of the cells (10, 14, 29). A dense intercellular matrix is expressed in multicellular spheroids (15). In contrast to the flattened cells in monolayer culture, those in multicellular spheroids have a rounded shape similar to *in vivo* tumors. Altered cell shape and chromatin structure can influence radiosensitivity (16, 47). Because of these differences, the available data on the fractionation sensitivity of individual human tumors should also include data from three-dimensional model systems.

Small spheroids from 1000–1500 cells are fully oxygenated under the conditions of the spheroid control assay used (63). To keep the effect of repopulation on the radiation response small, the intervals between fractions were 6 h, and the overall treatment time was maximally 50 h. A time interval of 6 h is considered to be sufficient for a complete recovery between the fractions. Experiments on the kinetics of recovery from sublethal radiation damage in spheroids of the sarcoma line EF8 showed a recovery half-time of 112 min, using the growth delay end point (76). In addition, studies on recovery kinetics for the sarcoma lines ESS2 and EA1 resulted in half-times below 1 h using the spheroid control end points (data not shown). The spheroid control assay allows the determination of the α and β values for a number of human tumor cell lines with a precision similar to that of the colony formation test. In the study by Peacock *et al.* (42) involving 11 tumor cell lines, the standard errors of the α_{LDR} and β_{RR} values were 11 and 15% of the mean values, respectively, while in this study the percantage errors of the α and β values from the spheroid control assay were 13 and 12%.

Distribution of the (α, β) data pairs

It is not possible to derive mechanistic models merely from the distribution of the α and β values from a panel of human tumor cell lines because of the complex molecular steps leading to reproductive cell death. However, implications of existing models such as the Curtis LPL model (6) can be critically tested by analysis of the (α, β) data pair distribution. According to the high dose rate low-dose approximation of the LPL model, α depends on the rates of production of lethal (η_L) and potentially lethal lesions (η_{PL}) per unit dose. The latter are weighted by the term $\exp(-\epsilon_{PL} \cdot tr)$, where ϵ_{PL} is the repair rate of potentially lethal lesions and tr is the postirradiation time after which no more repair can occur. β depends on η_{PL}, on the rate of binary misrepair ϵ_{2PL}, and on $\exp(-\epsilon_{PL} \cdot tr)$. Heterogeneity in both the α and β values between the various human tumor cell lines cannot be simply due to the intertumor variability of η_L or ϵ_{2PL} alone, because this would only explain an intertumor variability in either α or β. Both survival curve parameters, α and β, however, show a considerable variability between tumor cell lines (Fig. 4a and b). If the human tumor lines were to differ the repair factor $\exp(-\epsilon_{PL} \cdot tr)$ while all other parameters remained constant, then β values would decrease with increasing α values. Taking only heterogeneity in η_{PL} into consideration, the β values should increase while the α values increase or remain constant. The large scatter between (α, β) data pairs, observed in the panel of 21 human tumor lines, does not point to a dominant effect of one single parameter on the α and β values but, instead, to the intercell line variability of several parameters. Peacock *et al.* (42) observed a stronger correlation between the α and β values for clonogenic cells of 11 human tumor cell lines obtained from low dose rate and split course fractionation schedules than seen in this study ($r = 0.83$, $p < 0.002$). Their data can be interpreted in favor of the hypothesis that the high α and β values of radiosensitive cells are due to the induction of high frequencies of lethal and potentially lethal lesions per unit dose (42).

Comparison of the α/β values for human tumor lines in the spheroid system with those for experimental tumors in vivo *and human tumors in the clinic*

A comparison of the α/β values for 21 human tumor lines using spheroid control *in situ* with those determined *in vivo* for murine tumors showed human tumors to have

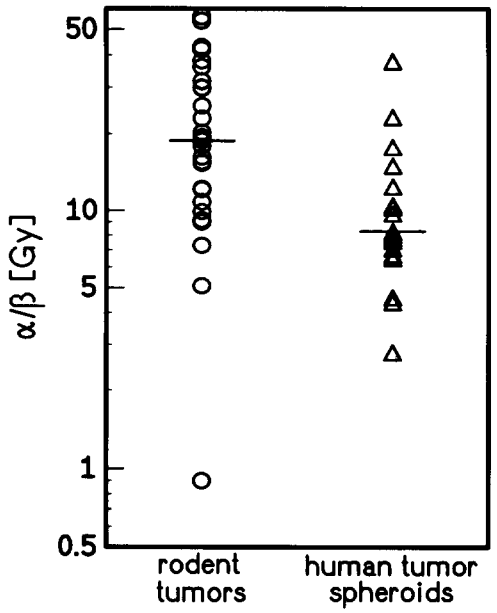

Fig. 9. Distributions of α/β values for rodent tumors *in vivo* and human tumor spheroids, as assayed by *in situ* end points. The 29 α/β values for 16 rodent tumors were obtained fom the literature, the 21 α/β values for different human tumor lines from this work. The rodent tumors had significantly higher α/β values (median: α/β = 18.8 Gy) than the human tumors (median: α/β = 8.3 Gy; $p < 0.001$, *U*-test).

significantly lower α/β values ($p = 0.0006$, *U*-test, Fig. 9). The murine tumor data used here for comparison were also determined from the *in vivo* end points tumor control or growth delay. Irradiation was performed under oxic or hypoxic conditions with short time fractionation schedules up to 20 fractions with intervals of 3–48 h, assuming complete recovery or with shorter intervals by taking incomplete recovery into account (17, 51, 80). In all, 29 α/β values for 16 different murine tumor lines were used for comparison. The median α/β value for the human tumor lines was 8.3 Gy, and for murine tumors 18.8 Gy. These differences could be related to a more pronounced selection in favor of undifferentiated murine tumor lines or to more rapid growth kinetics of murine tumor lines.

Evidence of a therapeutic gain in clinical Phase III trials is of decisive importance for the use of an altered fractionation schedule in the therapy of a certain tumor entity in the clinic. With increasing tumor size, the inter- and intratumoral heterogeneity also increases leading to a reduction in the steepness of the dose–tumor control relation (67). The γ_{50} value is a measure of the steepness of the tumor control curves (2). γ_{50} values of about 2–3 can be expected for head and neck carcinomas in early stages, and of about 0.5–1.5 for locally advanced tumors (74). The best data on the steepness of the dose–effect relations for local tumor control in the clinic stem from randomized dose escalation studies. From the local tumor control rates of the RTOG 83-13 study, the γ_{50} value is estimated to be 0.8 for locally advanced, inoperable squamous cell carcinomas of the upper respiratory and digestive tracts using hyperfractionated irradiation with 2 × 1.2 Gy per day (5). These flat dose–effect relations result in a low resolution of clinical studies in detection of fractionation effects on tumor control probability.

The well-conducted clinical Phase III trial on hyperfractionation of oropharynx carcinomas in Stages T2–3 with 160 patients per treatment arm, the EORTC 22791 study, resulted in a significant increase in local tumor control from 40 to 59% on increasing the total dose from 70 to 80.5 Gy and altering the daily dose fractions from 1 × 2.0 Gy to 2 × 1.15 Gy (22). The severe late side effects did not increase, therefore, resulting in a therapeutic gain. From this study, the mean α/β value for squamous cell carcinomas of the oropharynx can be estimated to be within the limits of 6.9 to > 100 Gy, as described in the Appendix. In this analysis, it is assumed among others that reoxygenation and repopulation of the tumors are not affected by the change in fractionation and that the γ_{50} value lies between 1 and 2 for the treated tumor population using conventional fractionation. This clinical study points to a low fractionation sensitivity of squamous cell carcinomas of the oropharynx. The experimental data from the spheroid model are compatible with this clinical result. The only squamous cell carcinoma line (FaDu) studied had the least fractionation sensitivity of all tumor lines investigated here with an α/β value of 38 (29–52) Gy. However, further studies are necessary to substantiate the hypothesis that the squamous cell carcinomas have a lower fractionation sensitivity than other tumor types.

Other clinical data on the fractionation sensitivity of human tumors are based on retrospective evaluation of patient collectives treated by single institutions. However, the selection criteria for the use of the different fractionation schedules are not necessarily independent of the prognosis of the disease (72). In particular, data were included in the determination of fractionation sensitivity from patients treated with subcurative palliative doses. In general, larger uncertainties in the determination of the α/β values have to be suspected from analyses of retrospective data than from Phase III trials. Unfortunately, the number of Phase III studies on hyperfractionation is presently still very low (8, 12, 22, 44, 53).

The fractionation sensitivities of individual human tumors measured here in a three-dimensional *in vitro* model were determined under oxic conditions with short time fractionation schedules to avoid repopulation during therapy. From a comparison of the experimental data with fractionation sensitivities of human tumors in the clinic, conclusions might be drawn about the effects of uncontrollable factors in the clinical situation such as reoxygenation and fractionation dependent repopulation. The α/β model has proved suitable for describing fractionation effects on spheroids derived from a number of human tumor lines. Because this model has passed this test, its

use for the description of the fractionation sensitivities of tumors in the clinical situation might be facilitated.

In this study, no correlation was observed between fractionation sensitivity and the SF2 or $SCD_{50\ 2\ Gy/fract}$ values as parameters for radiosensitivity or radioresponsiveness at clinical doses per fraction. Similarly, no significant relation was observed between the SF2 values from the colony formation test and the $\log(\alpha/\beta)$ values or the recovery ratios after fractionated high dose rate or low dose rate irradiation in three other studies using 10–13 cell lines in each and in a review involving 25 tumor cell lines (3, 4, 41, 42, 69). In particular, the malignant gliomas did not stand out with a distinctly large fractionation sensitivity. Therefore, high fractionation sensitivity or low α/β values do not turn out to be a dominant cause for the uniform radioresistance of malignant gliomas seen in the clinic. Despite the heterogeneity in the α/β values between the tumor cell lines, there is a close correlation between the $SCD_{50\ 1\ fract}$ and $SCD_{50\ 8\ fract}$ or the $SCD_{50\ 1\ fract}$ and $SCD_{50\ 2\ Gy/fract}$ values. Differences in the cellular survival rate after a certain dose, for example 2 Gy, and stem cell rate lead to such large differences in the radioresponsiveness of spheroids of various cell lines that the rank order of cell lines can hardly be altered over a wide range through a change in fractionation.

α/β values and G2 blockage

In this study, a good correlation was found between the blockage in the G2 phase and the $\log(\alpha/\beta)$ values ($r = 0.72$, $p = 0.004$, Fig. 7). This correlation becomes even better ($r = 0.90$, $p < 0.001$) if one does not include the data points of the line with the largest and smallest α/β value in the analysis, the only squamous cell carcinoma line (FaDu) and the paraganglioma line EPG1 which, according to histopathological criteria, shows resemblance to melanomas. A similar relationship between the G2 blockage and the α/β values was observed by Knox *et al.* (28) in three mammalian tumor cell lines. Whether the accumulation of cells in the G2 phase is causally responsible for the higher α/β values of the corresponding cell lines, or whether both factors only co-vary together due to the effect of a further parameter, such as tumor differentiation with different underlying tumor induction steps (65) cannot be definitely decided from the overall response of spheroids to the different fractionation schedules. However, this differentiation is not of decisive importance if one considers the increase in the G2/M parameter after fractionated irradiation only as predictive information about the fractionation sensitivity of the tumor lines in the spheroid model system. A further clarification, whether the α/β value is an intrinsic constant of the corresponding cell line, or causally dependent on cell cycling status, is beyond the scope of this work, which is restricted to the determination of the fractionation sensitivity of human tumor cell lines in the three-dimensional spheroid system.

Deviations from the predictions of the multifraction $\alpha\beta$ model for homogeneous cell populations assuming complete recovery between dose fractions or from the incomplete repair model (71) have also been observed in *in vitro* experiments with human tumor cell lines. In all these studies, the radiation effect was quantified using the colony formation test. These studies include observations of an inverse dose–rate effect at low dose rate, of differences between exponential and plateau phase cells in dependence of isoeffective doses on dose rate or dose fractionation in the absence of significant repopulation during treatment, or of an increase in the radiation effect per fraction with increasing number of previous dose fractions (11, 18, 27, 34, 36, 39, 40, 48, 56, 75, 78). The following have been identified as the mechanisms for these deviations: an alteration in the α/β value dependent on the cell cycle phase (19, 79) and repair exhaustion (11, 18, 36, 48), induced repair (31), differing random heterogeneities within the cell populations, which can reduce the curvature of the survival curves (55, 82), and redistributions over the cell cycle phases of different radiosensitivity with a sensitization of the population through accumulation in G2 phase (27, 34, 39, 40, 56, 75). However, in human tumor cells, the G2 phase is not a particularly uniform radiosensitive phase of the cell cycle, as shown from the few studies on human tumor cell lines (43, 45, 70, 76). In particular, tumor cells were found to be more radiosensitive in the G1 than in the G2 phase in those studies using centrifugal elutriation for cell synchronization (45, 76). Even with a knowledge of the dependence of radiosensitivity on the cell cycle phase distribution, it is not directly possible to draw conclusions about the changes in mean radiosensitivity of the surviving stem cell population during fractionated irradiation from changes in the cell cycle distribution of the whole cell population, because the stem cells only represent a small minority compared to the cells doomed to die (58). In this study, the linear quadratic model has adequately described the fractionation effect on spheroid control for schedules with 1 to 8 fractions. There were no indications of changes in the mean α values as a measure of radiosensitivity after the application of ≥ 2 or ≥ 4 fractions.

If the fractionation dependence of human tumor cells is only studied in one *in vitro* model system, the spheroid system stands out from the following properties. The cell–cell contact in the three-dimensional spheroid system can protect against radiation effects on cell cycle progression and can directly influence the recovery from sublethal damage (10, 14, 29, 49). The fact that the proliferation kinetics of human tumors are better reproduced in multicellular spheroids than in exponential or plateau-phase monolayers can be shown from the comparison of volume doubling times of multicellular spheroids with the potential tumor doubling times of human tumors *in vivo*. The latter are determined with BrdU labeling methods. The median spheroid volume doubling time for the 10 malignant glioma lines was 9.8 days, that for the 5 soft tissue

sarcoma lines was 6.7 days. The mean potential tumor doubling times (T_{pot}) for both tumor entities can be estimated to be 6–7 days, based on the *in vivo* bromodeoxyuridine labeling indices (LI) calculated according to the equation $T_{pot} = T_s \cdot 1/LI$, assuming a mean S-phase duration (T_s) of 11 h and the value 1 for the parameter l as a measure of the age distribution of the cells in the cell cycle. The median BrdU labeling index for 147 malignant gliomas was 6.9 (< 1.0–30.5)% (23), and 7.8 (0.2–32)% for 27 soft tissue sarcomas (37).

α/β values and tumor differentiation

In this study, apart from undifferentiated tumor lines, cell lines of human gliomas, sarcomas, and carcinomas classified differentiated were investigated. In the analysis of variance with the classification variables tumor type and tumor differentiation, only the effect of tumor differentiation was found to be significant. The fractionation sensitivity of undifferentiated, rapidly growing tumors was lower than that seen in differentiated, slowly growing tumors. A therapeutic gain is to be expected for these fast proliferating, undifferentiated tumors both with hyperfractionation as well as acceleration. The differentiated tumor lines had fractionation sensitivities overlapping the range of values for late reacting normal tissues.

APPENDIX

Radiobiological Models

Assuming complete recovery between dose fractions, the linear-quadratic model assuming Poisson statistics according to

$$p = \exp(-k \cdot \exp(-\alpha \cdot D - \beta \cdot D^2/n)) \quad \text{(Eq. 1)}$$

has been fitted to the observed frequencies of controlled spheroids in all dose groups of the different fractionation schedules using the maximum likelihood method. Here, p is the probability of spheroid control after the total dose D given in n fractions, α and β are the survival curve parameters, and k is the number of stem cells per spheroid. The deviations of the observed numbers of controlled spheroids in the different dose groups from those predicted by the model were analyzed by a χ^2 test (7), showing no significant deviations of the spheroid control data from the predictions of the model for any of the 21 cell lines in this study.

In addition, it was determined whether a more complex model allowing a change in the radiation sensitivity parameter α during fractionated radiotherapy better describes the spheroid control data than the α/β model according to Eq. 1. Therefore, the α-modifying function $\delta(n)$ was introduced

$$p = \exp(-k \cdot (\exp(-\delta(n) \cdot \alpha \cdot D - \beta \cdot D^2/n))) \quad \text{(Eq. 2)}$$

$\delta(n)$ was taken as a step function dependent on the number of fractions of the respective schedule adequate for the resolution of the experiments. Thereby, $\delta(n)$ was fixed to the value of 1 either only for the single dose [$\delta_1(n) = 1$, for $n = 1$] or also for the split course arms [$\delta_{1,2}(n) = 1$, for $n = 1,2$] of the respective experiment. For all other fractionation arms with $n > 1$ or $n > 2$, $\delta_1(n)$ or $\delta_{1,2}(n)$ could take a free value estimated according to the maximum likelihood method. If the free value of $\delta(n)$ is significantly different from 1, this is an indication of a change in the mean radiosensitivity with increasing numbers of fractions. This effect could be caused by alterations in the cell cycle distribution or an exhaustion of recovery capacity with increasing numbers of fractions (9, 36). This free part of $\delta(n)$ was not significantly different from 1 for the 21 tumor cell lines studied in the spheroid model. The mean $\delta_1(n > 1)$ or $\delta_{1,2}(n > 2)$ values and their standard deviation for all 21 cell lines were 0.99 $\pm$ 0.25 or 1.04 $\pm$ 0.12, respectively.

Furthermore, the goodness of the Poisson link function for describing the spheroid control data together with the α/β model was compared with the logistic link function. The log-likelihood values for the Poisson model were slightly smaller for 12 lines and slightly larger for nine cell lines than those obtained with the logistic model. Significant differences between the parameter estimates were not observed with both methods.

Estimation of the α/β ratio from the tumor control rates of clinical trials

The α/β ratio and its confidence limits were derived using the isoeffect relation of the α/β model and the definition of the γ_{50} value. According to the linear quadratic model (81), Eq. 13 applies to isoeffective fractionation schedules reaching tumor control probabilities p1 = p2 with total doses D1 and D2, given with doses d1 and d2 per fraction:

$$D1/D2 = (\alpha/\beta + d2)/(\alpha/\beta + d1) \quad \text{(Eq. 3)}$$

Different tumor control probabilities p1 and p3 after total doses D1 and D3, which should be around p = 50%, are interconnected by Eq. 4 for each fractionation schedule (2):

$$\gamma_{50} \approx (p3 - p1) \cdot D1/(D3 - D1) \quad \text{(Eq. 4)}$$

By combining Eqs. 3 and 4, the α/β ratio can be determined from the observed control rates p2 and p3 of two nonisoeffective fractionation schedules with total doses D2 and D3 given with doses per fraction of d2 and d1

$$\alpha/\beta = (d2 \cdot D2 - d1 \cdot D3/((p3 - p2)/\gamma_{50} + 1)))/(D3/((p3 - p2)/\gamma_{50} + 1) - D2) \quad \text{(Eq. 5)}$$

With 160 patients per treatment arm and a tumor control probability between 40 and 60%, the standard error of the 5-year locoregional control rate can be estimated to be ±4%, according to Greenwood's formula (24). After that, the mean and the 95% confidence limits for the α/β values for oropharynx carcinomas can be estimated from the hyperfractionation trial 22791 of the EORTC (22). The loco-regional tumor control rates at 5 years were 59 und 40% after total doses of 80.5 and 70 Gy given with 1.15 Gy or 2.0 Gy per fraction. The estimated α/β values were > 100 (12.6 to > 100) Gy, assuming a γ_{50} value of 1. For γ_{50} values of 2 or 3, the α/β estimates are about 19.7 (6.9 to > 100) Gy and 9.9 (5.9–24.0) Gy, respectively.

Relation between the SCD_{50} values for fractionation schedules with one and eight fractions

From the isoeffect relation of the α/β model according to Eq. 3, it follows:

$$SCD_{50\,8fract} = -4\cdot\alpha/\beta + \sqrt{16\cdot\alpha/\beta^2 + 8\cdot SCD_{50\,1fract}(\alpha/\beta + SCD_{50\,1fract})} \quad \text{(Eq. 6)}$$

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