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Dose dependence of the growth rate of multicellular tumour spheroids after irradiation

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Abstract. The present study investigated differences in the growth rate of multicellular tumour spheroids of the MCF-7 line of human breast cancer before and after their irradiation. Growth of the spheroids was analysed according to a model based on a Gompertz function. In this model, normalization to a common initial volume is achieved in a way that enables meaningful comparisons to be made between the results obtained for each spheroid. For irradiated spheroids the model includes an additional term to take account of sterilized cells. We found that the growth rate observed before irradiation is not fully recovered by irradiated spheroids and that growth recovery reduces with higher irradiation doses. Surviving fractions obtained at doses below 3 Gy are comparable with those found in clonogenic assays on spheroids of the same cellular line. At larger doses, discrepancies between the different studies are considerable.

Multicellular tumour spheroids (MTS) have attracted considerable attention [1–3] in recent years, largely because they mimic *in vitro* the micrometastases and growth of real tumours. MTS are composed of mixed cell populations and permit description of the avascular stage of tumour development. Thus, MTS are highly relevant to adjuvant therapy and are considered a good scenario to investigate tumour biology and to evaluate effects of different therapies.

Much of the work in this field has focused on the determination of cell radiosensitivity, expressed in terms of the cell surviving fraction (SF). This appears to be one of the most important parameters for treatment purposes because the radiosensitivity of human tumour cells in culture [4–6] and the radiation response of MTS [7] have been correlated with the clinical radiocurability of the corresponding tumour type.

However, the use of irradiated MTS to obtain the SF remains controversial. The common assumption of many studies is that irradiated MTS recover the growth rate of untreated MTS at some time after irradiation. This time period, known as the growth delay (GD), has been variously considered the time required for treated MTS to regrow to four-fold [8] or eight-fold [9, 10] their initial volume. If the growth recovery assumption is correct, these discrepancies have little relevance because GD will be independent of MTS volume. In addition, some studies [8] showed that GD may not be appropriate to compare the radiosensitivity of tumours of different sizes.

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Other methodologies [11] obtain the SF as the ratio between the zero time intercept of the extrapolated linear part (on a logarithmic scale) of the regrowth curve and the initial volume. However, the regrowth curve does not generally follow an exponential shape, so that it is often difficult to unequivocally define the linear part of the curve. In addition, the volumes of different MTS do not undergo any kind of normalization, making comparisons between them inadequate and therefore negating the significance of the characteristic parameters of the growth model.

The present study aimed to test the hypothesis that the growth rate recovers after irradiation, using a novel method to investigate both untreated and irradiated MTS. The MTS are considered individually by a technique that avoids the usual procedure of averaging the volumes of the different MTS at each time point. Thus, the growth curve of each spheroid can be normalized to a given initial volume, allowing valid comparisons to be made. The method also takes account of the coexistence of two different cell populations after irradiation; sterilized and surviving cells. The former do not proliferate and it is the growth rate of the latter that is of interest. Finally, the SF is directly obtained by fitting a mathematical model to the experimental data, so that GD or extrapolation procedures are unnecessary.

Materials and methods

Cell culture

Cells of the MCF-7 human breast cancer line established by Soule et al [12] and obtained from Dr G Leclercq, Institut Jules Bordet, Brussels were used. Cells were grown routinely in minimum essential medium supplemented with 10% foetal bovine serum. Monolayer cultures were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Cells used to develop the spheroids were obtained

by trypsinization from exponentially growing monolayers and cultured in the medium described above.

Spheroid initiation

Spheroid cultures were initiated by seeding 1000–1500 cells into each well of six 48-well plates. Each well was previously coated with a thin layer of 1% agar (Bacto agar; Difco, Detroit, MI) and contained standard culture medium. The six plates were agitated for 18–20 h under the conditions described above for the monolayer cultures. In this way, a single spheroid of approximately 100 μm diameter was obtained in each well. Once the MTS were formed, the medium was changed every 3 days.

Ionizing radiation

The diameter of the MCF-7 spheroids was measured every 1–2 days using an inverted phase contrast microscope. The volume of each spheroid was calculated as $V=(\pi d^3)/6$, where d is diameter. At the time of irradiation the spheroids presented diameters of 220–280 μm. One of the six plates was kept as a control and the other five were exposed to single doses of 18 MV X-rays (range 1–5 Gy) using an electron linear accelerator (Mevatron KDS; Siemens, Erlangen, Germany). Electronic equilibrium was ensured by placing the biological material between solid water slides (RW3 of PTW Freiburg). The total depth was 3 cm above and 20 cm below the samples.

Spheroid selection

Only spherical spheroids were considered in this study. In previous studies [13, 14] our group estimated volumes by assuming spheroids to be ellipsoidal and measuring two perpendicular diameters. In the present work all spheroids with evident asymmetry in the initial growth stages were discarded. Spheroids also become asymmetric when they grow to considerable size. In order to maximize the number of spheroids in the sample, time limits for their evaluation were set: 19 days for the control spheroids and those exposed to 1 Gy or 2 Gy, and 21 days for those exposed to 3–5 Gy.

In order to guarantee homogeneity for analysis, only spheroids that showed no growth problems, *i.e.* they were not the result of the fusion of two or more initial spheroids, included no agar fibres or other foreign matter and were not in a contaminated well, were considered.

A very small number of spheroids failed to regrow after irradiation and were excluded from analysis.

At the time of irradiation, the MTS had a homogeneous composition and showed no necrotic nuclei, fulfilling the condition that the relationship between spheroid volume and number of cells be proportional. 18 control MTS and 15, 28, 22, 20 and 14 MTS irradiated with 1 Gy, 2 Gy, 3 Gy, 4 Gy and 5 Gy, respectively, were considered valid for inclusion in the study.

Mathematical approach

The mathematical approach used permits analysis of growth data for both control and irradiated MTS.

Control spheroids

In order to test the assumption that treated MTS recover the growth rate of untreated MTS at some time after irradiation, it is necessary to correctly determine the parameters of the untreated spheroids. The usual procedure [9, 11, 15] requires two steps. First, the mean volume $\bar{V}$ of the spheroids at each time t_j is calculated as:

$$\bar{V}(t_j)=\frac{1}{n}\sum_{i=1}^{n} V_i(t_j) \quad j=1,\ldots,k \tag{1}$$

where n is the number of spheroids included in the sample, $V_i(t_j)$ is the measured volume for the i-th spheroid at the time t_j and k is the number of time points at which the volumes are measured. These mean values are then fitted to a given sigmoid function.

As previously mentioned, valid comparisons are compromised by differences in volume of different spheroids at the time of first measurement, and it is necessary to normalise data to a common initial volume (CIV). Some authors [14] carried out this normalization by dividing the volume data of each spheroid by V_0, the corresponding volume at the initial time point. The resulting data were fitted to the model function for each spheroid individually, and the corresponding mean parameters were evaluated to obtain a "standard growth curve". However, this procedure is not adequate because it does not permit comparison between different MTS at the same growth stage, as we shall demonstrate below. The present paper proposes a novel approach to CIV normalization that addresses this issue and obtains meaningful characteristic parameters. Thus, each control spheroid in the sample was considered individually and the increase in its volume described using a Gompertz function, following a previously published procedure [13], which relates the volume at a given time t_1 to the volume of the MTS at a previous time t_0:

$$V(t_1)=V(t_0)\ \exp\{\frac{\lambda}{a}[1-e^{-a(t_1-t_0)}]\} \tag{2}$$

Here λ is the initial specific growth rate and a is the proportional rate of decay of λ.

This formula was developed by Gompertz [16] for actuarial assessment of England's population. A century later this formula was proposed as a possible model for biological growth [17, 18]. In 1964 it was empirically found that Gompertz formula sucessfully described the growth of individual organisms and tumours [19].

The normalization to CIV proposed here is based on the assumption that the growth of each spheroid occurs in two stages. In the first stage, which is not actually observed, the MTS develops from CIV V_{CI} to the volume observed at the time of the first measurement $t=0$, V_0 after time T has elapsed.

$$V(t=0)\equiv V_0=V_{CI}\ \exp\{\frac{\lambda}{a}[1-e^{-aT}]\} \tag{3}$$

It is important to note that time T is particular to each spheroid and can be readily calculated with this equation when the parameters have been determined.

In the second stage, which is observed, the MTS grows from volume V_0 to that measured at time t. Hence, the total time elapsed from the CIV to the measured volume at the time t is $t+T$, giving:

$$\begin{aligned} V(t+T) &= V_{CI}\ \exp\left\{\frac{\lambda}{a}\left[1-e^{-a(t+T)}\right]\right\} \\ &= V_{CI}\ \exp\left\{\frac{\lambda}{a}\left[1-e^{-aT}\right]\right\}\ \exp\left\{\frac{\lambda}{a}e^{-aT}\left[1-e^{-at}\right]\right\} \\ &= V_0\ \exp\left\{\left(\frac{\lambda}{a}-\ln\frac{V_0}{V_{CI}}\right)\left[1-e^{-at}\right]\right\} \\ &= V_0\ \exp\left\{\frac{\tilde{\lambda}}{a}\left[1-e^{-at}\right]\right\} \end{aligned} \tag{4}$$

Thus, the normalization of all the spheroid growth curves to the CIV is characterized, in practice, by a new value of parameter λ of the Gompertz model:

$$\tilde{\lambda} = \lambda - a\ln\frac{V_0}{V_{CI}} \tag{5}$$

Conversely, parameter a is independent of the normalization procedure. For convenience we used $V_{CI} = 10^6\ \mu m^3$, which was smaller than all the volumes measured. However, any other choice of value would not have modified the results obtained in our analysis.

Once this normalization is performed, MTS volumes can be compared. The growth data of each spheroid are fitted by means of Equation (4), and the parameters characterizing the growth of the control MTS are obtained as the mean value of parameters λ and a for each spheroid.

Irradiated spheroids

Growth curves for irradiated spheroids have specific characteristics that must be incorporated into the theoretical model that describes them:

(1) Once the spheroid is irradiated, its volume either increases slowly, remains constant or diminishes, and the spheroid can subsequently regrow or the regrowth can fail. The number of failures increases with higher doses [9, 11, 14, 15].
(2) Once the stagnation or regression phase is overcome, the slope of the growth curve appears to be similar to that shown by the control spheroids, at least for a range of doses (the range depends on the cell line under study) [9, 14, 15]. However, more rigorous study of the data casts doubt on this apparent similarity.

These characteristics justify the definition of the GD as the difference between the times needed for control and irradiated spheroids to reach a certain volume. However, this volume has been variously proposed as eight-fold [9, 15], five-fold [20, 21] and four-fold [8] the initial volume, and there are no objective criteria to choose between these proposals.

Our method avoids this issue, based on a previously reported approach to the problem [22]. It is assumed that two sets of cells coexist after irradiation: surviving cells, responsible for the regrowth of the spheroids; and sterilized cells, including dead cells and those that have lost their proliferation capacity. In our model, the total volume of the irradiated spheroids is expressed as the sum of the volumes of these two subsystems:

$$V(t) = V^{surv}(t) + V^{ster}(t) \tag{6}$$

where V^{surv} is the volume of surviving cells and V^{ster} is the volume of sterilized cells.

We assume that growth of surviving cells follows the same pattern (the Gompertz model) as growth of cells in control spheroids:

$$V^{surv}(t) = V_0^{surv}\ \exp\left\{\frac{\lambda}{a}\left[1-e^{-at}\right]\right\} \tag{7}$$

We further assume that the sterilized cells will maintain or decrease the volume they show at the time of irradiation. In our model, cell loss is negligible and data are compatible with a virtually constant value for $V^{ster}(t)$. Indeed we observed no appreciable loss of cellular material during the time the spheroids were evaluated.

Taking the above into account, the following model was used for analysis of the irradiated MTS:

$$\begin{aligned} V(t) = V_0^{surv}\ &\exp\left\{\left(\frac{\lambda}{a}-\ln\frac{V_0^{surv}}{V_{CI}}\right)\left[1-e^{-at}\right]\right\} \\ &+\left(V_0 - V_0^{surv}\right) \end{aligned} \tag{8}$$

the moment of the first measurement V_0 being very close in time to irradiation. We considered that $V(t=0) = V_0$. The normalization to CIV is included in the procedure, as for control spheroids. This equation is used to analyse growth data of irradiated spheroids and to determine whether characteristic parameters remain the same as for control MTS.

As mentioned above, our spheroid selection procedure allows us to consider volume of a spheroid and the number of clonogenic cells it contains to be proportional [23]. Hence, the fitting procedure directly yields the SF(s):

$$s = \frac{V_0^{surv}}{V_0} \tag{9}$$

which allows us to obtain the radiosensitivity parameters of the cell population.

It is very important to note that normalization to CIV, a basic element of our approach, does not modify the SF values found, because it affects only the λ parameter and leaves both V_0 and V_0^{surv} unchanged.

Statistical methods

Uncertainties in measurement of spheroid volumes must be considered. Sources of uncertainty in this experiment include loss of the spherical shape of the cellular aggregates and the effects of periodic changes in the culture medium, among others. However, with our method, only uncertainties linked to instrumental error can be evaluated. The smallest division in the ocular of the microscope was 10 μm, so that uncertainty in the volume $u(V)$ was $u(V) = 5\pi d^2\sqrt{2/3}$ (in μm^3) with d given in micrometres, assuming a uniform distribution for the uncertainty of the measured diameter [24].

The experimental data obtained were fitted to Equation (4) for control spheroids and Equation (8) for irradiated spheroids. The Levenberg–Marquadt method was used as the fitting procedure. This method provides the parameters of the model function by giving the fit with the smallest χ^2.

Table 1. Values of the parameters of interest for the control multicellular tumour spheroids obtained in the different approaches. The last column corresponds to the value of the initial specific growth rate λ of the Gompertz equation renormalized to common initial volume (CIV). Uncertainties correspond to one standard deviation

	λ [day^{-1}]	a [day^{-1}]	λ (renormalized) [day^{-1}]
Authors' approach	0.64 ± 0.01	0.086 ± 0.002	—
Without CIV	0.461 ± 0.006	0.086 ± 0.002	0.638 ± 0.008
Mean volume	0.459 ± 0.001	0.0864 ± 0.0001	0.64 ± 0.01

a, proportional rate of decay.

Results and discussion

Control spheroids

Parameters for control spheroids determined by this method are shown in Table 1. Figure 1a depicts the fit obtained for a representative spheroid. For all the MTS in the control sample we obtained a mean χ^2 per degree of freedom of 1.3 ± 0.2, where the uncertainty corresponds to one standard deviation.

The normalization to CIV is one of the most important elements of our approach to the analysis of MTS growth and we compared our results with those obtained by the usual methodologies, which do not include this element. Analysis using our method but without including the normalization step was performed and a value of λ (Table 1) (0.461 ± 0.006 days^{-1}) very similar to that determined by fitting the mean volumes given by Equation (1) was found (Table 1). Inclusion of the normalization procedure had a clear impact on the value of λ, which was markedly different with our method, whereas the value of a was the same. However, the differences observed here derive only from the different initial volumes corresponding to each case. To obtain a more meaningful comparison, Equation (5) was used to obtain the λ parameter corresponding to values found using procedures that did not use normalization, considering mean volumes as volume V_0 and normalizing again to $V_{CI}=10^6$ μm^3. Results (Table 1) were comparable with the value of λ determined by our new procedure.

Dose-dependence of growth rate

The principle issue with treated MTS is whether the growth rate recovers or not. In Figure 1b–f, volumes of a representative spheroid for each of the doses administered are shown and the results of fitting Equation (8) to the

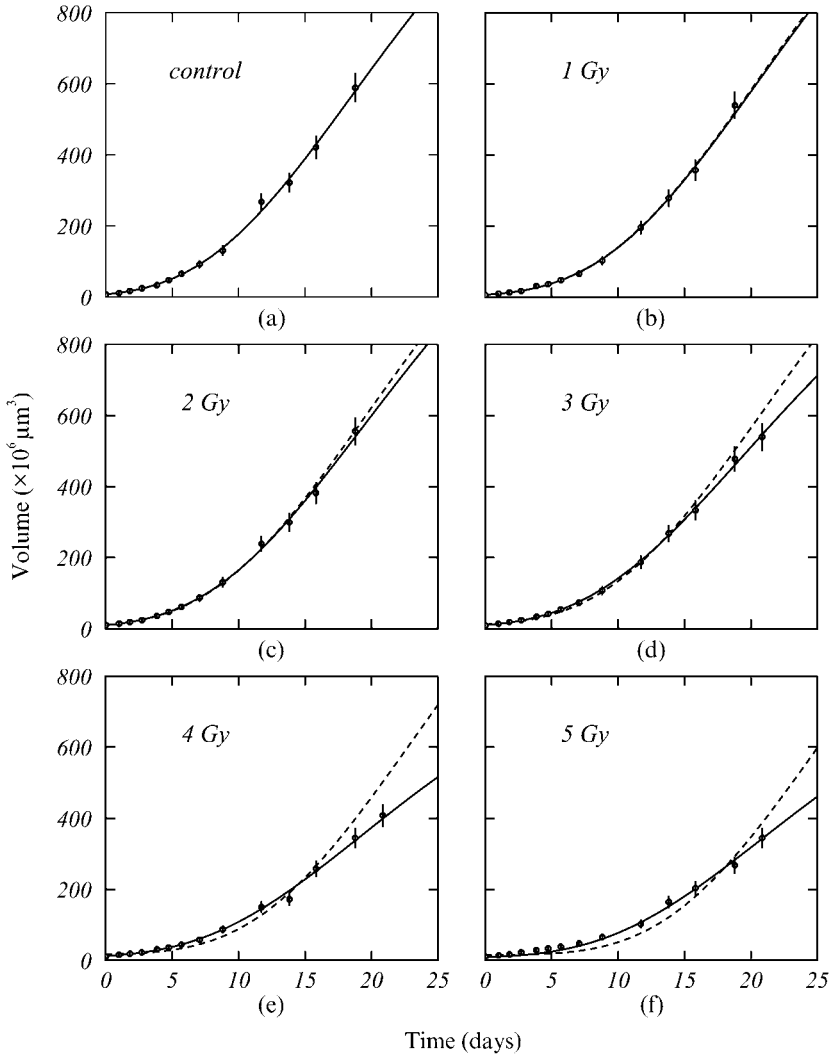

Figure 1. Representative examples of control (a) and irradiated spheroids (b–f). Uncertainties correspond to 2 standard deviations. ---, fits obtained by fixing the initial specific growth rate and the proportional rate of decay to the values obtained for the control spheroids; —, fits obtained by fixing only the proportional rate of decay to the values obtained for the control spheroids.

measured volumes plotted, considering the parameters λ and a to be the same as those obtained for the control MTS. In general, the fit was rather poor (mean χ^2 per degree of freedom ranged from 4.3 ± 0.5 for 1 Gy to 27 ± 1 for 5 Gy). This finding appears to challenge the common assumption of growth rate recovery after irradiation.

We investigated this issue further by directly fitting the data to the model function, Equation (8). In principle, if both λ and a are considered as free parameters, the function includes four parameters rather than the three included for control spheroids in Equation (4), which complicates the fit. We determined that the optimal procedure is to establish parameter a as the mean value obtained in analysis of the control sample (a=0.086 days^{-1}), bearing in mind that a was not changed by the CIV normalization procedure, as shown above. Thus, we tested the growth rate recovery assumption by studying the variations of parameter λ. The fit found with this method was considerably better than those shown above; mean χ^2 per degree of freedom ranged from 1.7 ± 0.4 for 1 Gy to 3.8 ± 0.5 for 5 Gy. Some examples are depicted in Figure 1. Figure 2 shows the variation in λ values obtained in this analysis according to dose; uncertainties correspond to one standard deviation. It can be concluded that growth rate of the control spheroids was not fully recovered by the irradiated spheroids. Our analysis procedure reveals that parameter λ decreased linearly with increased dose. Figure 2a displays the linear regression that describes the dependence of λ on dose d. The corresponding characteristic parameters are given in Table 2. Thus, we can state that growth of the exposed spheroids was perturbed by irradiation.

As in the case of the control spheroids, the influence of the CIV normalization step included in the approach used was investigated. For this purpose, the spheroids were also analysed individually but without normalization. The results obtained are shown in Figure 2b. Uncertainties are considerably larger than those found when the normalization to CIV is included. The value of λ obtained for control spheroids was very similar to the values found for irradiated MTS at all doses administered. This can be verified by the corresponding linear regression of these data, also plotted in the Figure 2, which produced the results shown in Table 2. Despite the poor values of linear correlation obtained, the value of λ appears to be practically independent of dose. This is a consequence of the mixture of MTS at different growth stages, which is inherent to this procedure.

Figure 2b also depicts the values of λ parameter found when results were renormalized by means of Equation (5), using mean volumes obtained in the fitting procedure. As in the case of control spheroids, these new values were similar to those obtained with our procedure. The main difference is that the uncertainties were clearly larger. The corresponding linear regression, whose parameters are given in Table 2, was also plotted. The results again showed the diminution of the parameter λ with increased dose. However, the fact that uncertainties are now larger than in our approach makes the decrease of λ less evident. The advantage of our approach is clear because normalization to CIV performed for each MTS individually produces very accurate results.

Our results do not support the commonly accepted hypothesis of recovery of pre-irradiation growth rate. This assumption is based on the apparently similar slopes shown by the growth curves of both treated and untreated spheroids. We have seen that values of the parameter λ found with the usual fitting procedures appear to support the growth rate recovery hypothesis. However, we have

Table 2. Results of the linear regression $\lambda(d)=md+n$ of the initial specific growth rate λ as a function of dose d with and without the normalization to common initial volume (CIV). The bottom row gives the results obtained after a renormalization of the latter data. The parameters characterizing the regression m and n and the correlation coefficient r are given. Uncertainties correspond to one standard deviation

	m [days^{-1} Gy^{-1}]	n [days^{-1}]	r
Authors' approach	-0.012 ± 0.001	0.646 ± 0.004	-0.982
Without CIV	0.002 ± 0.002	0.463 ± 0.005	0.359
Renormalized	-0.008 ± 0.003	0.641 ± 0.007	-0.971

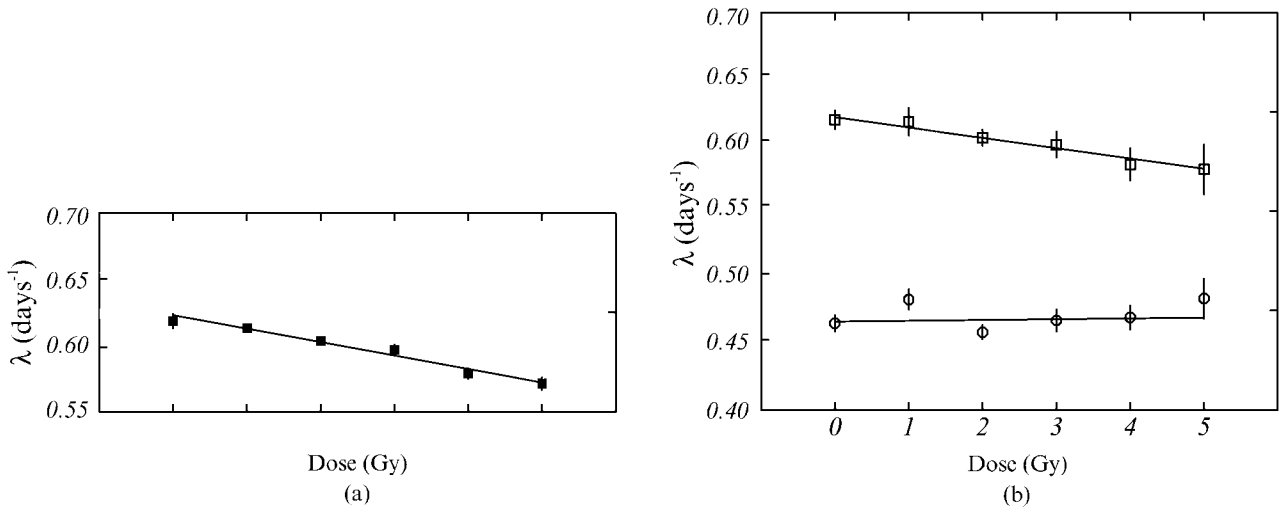

Figure 2. Variation of the initial specific growth rate λ with dose administered. Uncertainties correspond to one standard deviation. (a) Linear fit corresponding to the method used in this study. (b) Linear fit of results obtained in a similar analysis. ○, results obtained without normalization; □, results obtained from the latter analysis and normalized to a common initial volume, as discussed in the text.

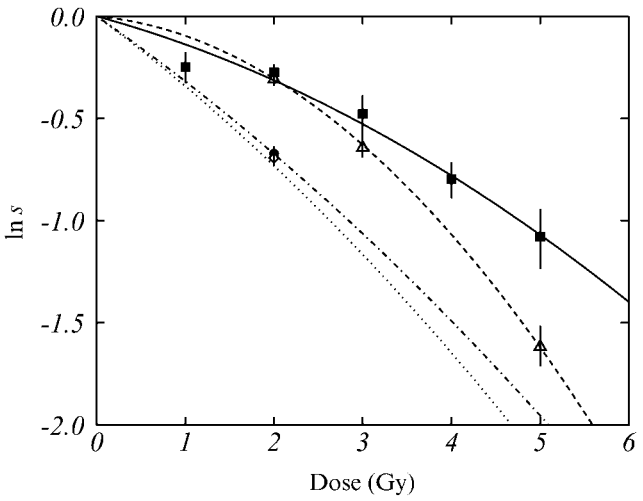

Figure 3. The natural logarithm of the surviving fraction (s) as a function of the imparted dose. ■, results of the method used in this study; —, corresponding regression to the linear-quadratic model; -.-., results quoted by Villalobos et al [21] (●, 2 Gy dose); …, linear-quadratic fit obtained by Núñez et al [28] (◇, 2 Gy dose); △ and - - -, results corresponding to reanalysis of data of Villalobos et al [21]. Uncertainties correspond to one standard deviation.

unequivocally demonstrated that after normalization to the CIV, λ varies with dose. It is important to state that this variation of λ cannot be considered an effect of the "volume change" inherent to the CIV normalization because by fixing the same initial volume for all the spheroids, growth data can be compared and conclusions are meaningful.

Surviving fraction

Figure 3 shows the values of the SF obtained in our regrowth assay using Equation (9). Following the standard approach to describe this SF, we fitted the data obtained by means of the linear-quadratic model:

$$-\ln s = \alpha d + \beta d^2$$

where the parameters α and β are characteristic of the cell line considered. This model has been widely used [26, 27] and despite its simplicity, provides a satisfactory description of the survival curve of a homogeneous cell population.

As can be seen, the fit was very good except for dose 1 Gy. Given the accuracy of our method, this suggests the need for further investigation of the low dose region of the survival curve.

Parameters obtained in the fit to the linear-quadratic model in our case are shown in Table 3, where these results are compared with those obtained for the same cell line in a monolayer culture performed by Núñez et al [28] and those obtained previously by our group [21] for spheroid growth, both using clonogenic assays (CA). The values of the SF for a 2 Gy dose are also shown. Figure 3 also depicts this comparison. Results obtained in the two CA (monolayer and MTS) were very similar and differ from the present findings, even at low doses.

The difference in results between our regrowth assay and the CA of MTS performed by Villalobos et al [21] is surprising. We have reanalysed the latter data and performed some additional experiments of the same type for doses up to 5 Gy. The results of this new study are also shown in Table 3 and were closer to those obtained with our new approach. In particular, the SF for 2 Gy coincide. Nevertheless, the values of the parameters of the linear-quadratic model were considerably different, so that they cannot be calculated by means of the classic GD model. This is apparent in Figure 3, which shows large discrepancies at high doses between the results of our procedure and those of the repeat CA of MTS. We can conclude that cells organized as MTS are more resistant to radiation than are monolayer cultures. These results have also been supported by Olive et al [29].

It is important to note that the clonogenic and regrowth assays produce comparable results for doses up to 3 Gy, because CA are known to be generally simpler to develop than regrowth with MTS and subsequent analysis is less complicated.

Although GD is not necessary in the method used here, SF results were compared with those obtained when the current GD definitions are applied to the data from this experiment. The corresponding SF from the GD was calculated by modifying the equation given by Rofstad et al [20] for an exponential growth model, in order to obtain one appropriate to the Gompertz model. If a N_V-fold increase in initial volume is considered we have:

$$\ln s = \frac{\lambda}{a^2}\exp(-at)\left[\exp\left(-at_{\text{delay}}\right)-1\right]$$
$$= \left(\frac{\lambda}{a^2}-\ln N_V\right)\left[\exp\left(-at_{\text{delay}}\right)-1\right]$$

where t_{delay} represents GD.

Table 3. Comparison of α, β and the surviving fraction at 2 Gy, s(2 Gy), obtained in this work with those found in clonogenic assay (CA) for monolayer culture and for multicellular tumour spheroid (MTS) growth. The values obtained from the growth delay (GD) for 5- and 8-fold increase in initial volume are also given. Uncertainties correspond to one standard deviation. α and β are characteristic of the cell line considered

	α [Gy^{-1}]	β [Gy^{-2}]	s (2 Gy) (%)
CA monolayer (Núñez et al [28])	0.32 ± 0.02	0.023 ± 0.006	50 ± 2
CA MTS (Villalobos et al [21])	0.30 ± 0.10	0.018 ± 0.005	51
Regrowth MTS (present work)	0.12 ± 0.03	0.019 ± 0.008	76 ± 3
CA MTS (present work)	0.04 ± 0.03	0.057 ± 0.009	74 ± 3
GD 5-fold (present work)	0.11 ± 0.02	0.027 ± 0.004	71 ± 7
GD 8-fold (present work)	0.13 ± 0.01	0.033 ± 0.004	67 ± 9

The values of the parameters of the regression to the linear-quadratic model are given in Table 3. Differences between the values obtained with the two definitions of the GD are again clear. There is also an apparent difference with our results, mainly at high doses. Nevertheless, it is interesting to note how different methods provide similar values of SF for low doses. In any case, this comparison clarifies the indetermination in the SF produced by the GD approach.

Conclusions

The hypothesis of regrowth rate recovery by MTS after irradiation was investigated. An analysis of the growth data of multicellular spheroids that includes normalization to a common initial volume was developed, to allow meaningful comparison between the different spheroids. As a direct result, the model gives the surviving fraction of the sample.

This model was applied to spheroids of the MCF-7 line of human breast cancer. The most important finding was that normal growth rate is not fully recovered by the irradiated spheroids, and that the difference in growth rate is greater at higher doses. Both treated and control MTS show apparently similar growth rates when data are not normalized to common initial volume, which accounts for the commonly held assumption of recovery after irradiation. The fact that the growth rate is not fully recovered may be of clinical relevance in planning therapy protocols for patients.

The surviving fractions obtained are comparable with those found in different experiments with MTS of the same cellular line, provided dose is low. For higher doses, discrepancies between different experiments are considerable. At any rate, we can affirm that clonogenic assays are sufficiently accurate to provide the surviving fraction at doses below 3 Gy, and offer clear advantages in experimental procedure and data analysis.

The accuracy of the approach used is comparable with that usually achieved in clonogenic assays with spheroids, and the regrowth assay is a less disturbing method to analyse the surviving fraction. Nevertheless, the regrowth assay is both less laborious and requires lesser disturbance of the cells, making our novel methodology of special interest.

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