Cell Prolif. 2000, **33**, 219–229

Forecasting the growth of multicell tumour spheroids: implications for the dynamic growth of solid tumours

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(*Received* 18 *May* 1999; *revision accepted* 22 *December* 1999)

Abstract. The growth dynamics of multicell tumour spheroids (MTS) were analysed by means of mathematical techniques derived from signal processing theory. Volume *vs*. time trajectories of individual spheroids were fitted with the Gompertz growth equation and the residuals (i.e. experimental volume determinations minus calculated values by fitting) were analysed by fast fourier transform and power spectrum. Residuals were not randomly distributed around calculated growth trajectories demonstrating that the Gompertz model partially approximates the growth kinetics of three-dimensional tumour cell aggregates. Power spectra decreased with increasing frequency following a $1/f^{\delta}$ power-law. Our findings suggest the existence of a source of 'internal' variability driving the time-evolution of MTS growth. Based on these observations, a new stochastic Gompertzian-like mathematical model was developed which allowed us to forecast the growth of MTS. In this model, white noise is additively superimposed to the trend described by the Gompertz growth equation and integrated to mimic the observed intrinsic variability of MTS growth. A correlation was found between the intensity of the added noise and the particular upper limit of volume size reached by each spheroid within two MTS populations obtained with two different cell lines. The dynamic forces generating the growth variability of three-dimensional tumour cell aggregates also determine the fate of spheroid growth with a strong predictive significance. These findings suggest a new approach to measure tumour growth potential.

INTRODUCTION

Two main and important features characterize the growth of solid tumours: variability and saturation. Though not yet defined clearly, the meaning of tumour growth variability and of growth saturation arises from experimental observation. Variability means that tumour cells of the same histotype can produce tumour masses whose volume increases in time following

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heterogeneous kinetics so that, at a certain time, two tumours may have very different volumes. Saturation, instead, refers to the fact that a tumour mass can not grow indefinitely but, at a certain time and after a quasi-exponential growth phase, its size reaches an upper limit (also called asymptotic volume). Thus, the size of a tumour increases in time following an S-shaped growth curve which is best approximated by the Gompertz growth equation (Bajzer, Vuk–Pavlovic & Huzak, 1997):

$$V(t) = V(0)\exp\{\alpha/\beta[1 - \exp(-\beta t)]\} \quad (1)$$

where V(t) is the volume of a tumour at time t, V(0) is the initial volume and α and β are positive parameters.

Both growth variability and saturation are observed *in vivo* for experimental tumours in animals (Norton *et al*., 1976; Brunton & Wheldon, 1980; Steel, 1980; Norton, 1985; Demicheli *et al*., 1989), whereas it is still a matter of debate whether these features characterize the growth of solid tumours in humans (Hart, Shochat & Agur, 1998). When fitting tumour growth data with the Gompertz equation, the growth variability is reduced to a linear correlation between the two parameters α and β which holds for all tumours of the same histotype and appears to be species-specific (Norton *et al*., 1976; Brunton & Wheldon, 1980; Demicheli *et al*., 1989). This important result would establish a relation between growth variability and saturation since in the Gompertz growth model the asymptotic volume (V_∞) is related to the parameters α and β by the following:

$$V_\infty = \lim_{t \to \infty} (V(t)) = V(0)e^{\alpha/\beta} \quad (2)$$

In other words, different tumour masses would approach in time the same asymptotic volume irrespective of the initial conditions and hence of the individual growth kinetics (which can be highly variable).

However: (i) to date, the above theoretical prediction that different tumour masses would approach in time the same asymptotic volume has not been proven experimentally. Rather, analysis of tumour growth carried out for tumours grown in different species of mammals has revealed that the asymptotic volumes of tumours may vary over two orders of magnitude (Steel, 1980) in spite of the linear correlation found between Gompertzian parameters (Brunton & Wheldon, 1980). The origin of variability of asymptotic volumes is still not understood; (ii) both variability and saturation of tumour growth may have an important dynamic as well as biological meaning and therefore should be investigated in detail. For example, it could be hypothesized that the levels at which the volume of different tumours saturate in growth curves might be related to growth variability.

Understanding the growth of tumours may have profound clinical and biological implications. The designing of therapeutic strategies aimed at controlling neoplastic growth would benefit from a deeper knowledge of tumour growth dynamics and hence of the underlying biological laws. New information can be gathered by detailed study of tumour growth using modern methods of time series analysis. To do this, however, a large number of experimental determinations of tumour volume are needed. This is best accomplished *in vitro* using a three-dimensional tumour model (multicellular tumour spheroids, MTS) due to the limiting number of accurate volume determinations that can be obtained in patients or even in experimental animals.

MTS represents an experimental model with an intermediate complexity between solid tumour *in vivo* and traditional two-dimensional cell cultures (Sutherland, 1988). They share many biological properties in common with solid tumours, in particular gradients for nutrients and oxygen which rapidly decrease in the inner layers as a function of MTS size.

This leads to a gradient of cell proliferation with actively proliferating cells in the outer rims of an MTS and nonproliferating/quiescent or dying cells in the central core. At a certain critical size, the central core of an MTS becomes necrotic (Sutherland, 1988).

Since in MTS cells adhere to each other, they display molecular and physiological characteristics which mimic those of cells in tumour tissues which cannot be appreciated in 2D-cultures. Among these are: (i) expression of adhesion molecules (Waleh *et al*., 1994) (ii) production of an extracellular matrix (Nederman *et al*., 1984) (iii) expression of a multidrug-resistant phenotype (Durand, 1981; Sutherland, 1988). For these reasons, MTS have been extensively utilized in recent years to study the biology of tumours and to test novel antitumour treatments (Sutherland, 1988).

The growth kinetics of MTS also parallel those of *in vivo* tumours, and they have been found to obey the Gompertz growth model (Demicheli *et al*., 1989). Several biological and biophysical conclusions have been derived to explain why the Gompertz equation is the best descriptor of biological growth in general and of tumours and MTS growth in particular (Laird, 1964; Laird, 1969; Demicheli *et al*., 1989; Bajzer *et al*., 1997). However, we recently showed that when the growth trajectories of individual MTS are analysed using the Gompertz growth equation followed by signal analysis techniques, new growth patterns arise which consist of temporal quasi-periodic oscillations of MTS volume (Chignola *et al*., 1999). These patterns are not predicted by the Gompertz growth model.

In this study, we put forward our analysis of the growth patterns displayed by individual MTS. A mathematical model has been developed based on experimental data which allowed us to forecast the growth of MTS. A correlation between variability and volume saturation was also found. These results show that it is possible to predict the endpoint of MTS growth by analysing their individual growth dynamics. A new approach to forecast tumour progression is suggested.

MATERIALS AND METHODS

Generation of multicellular tumour spheroids

9L (rat glioblastoma) or U118 (human glioblastoma) cells were cultured at 37 °C in a 5% carbon dioxide atmosphere in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics, and passaged weekly. Spheroids were obtained by inoculating 10^6 cells in 10 ml of RPMI-FBS 10% in Petri dishes on a thin layer of agar [10 ml of a 0.75% (w/v) solution of agar in RPMI-FBS 10%]. Spheroids of about 200–250 μm diameter (approximately 2000–4000 cells per spheroid) were harvested by gentle repeated transfer with a micropipette of individual spheroids into the wells of a 24-well culture plate. MTS were then individually placed into the wells of a 24-well culture plate containing 1 ml of RPMI-FBS 10% on a layer of 1 ml of 0.75% (w/v) agar in the same medium. Every 7 days 0.5 ml of RPMI medium were gently removed from each well; the wells were then filled with the same amount of fresh medium. Spheroids were measured using a calibrated ocular micrometer on an inverted microscope. At best resolution, one unit of the metric scale corresponds to 34 μm. The longest spheroid diameter (D) and the perpendicular diameter (d) were measured; the volume (V) was calculated according to the formula $V = 4/3\pi r^3$, where $r = (Dd)^{1/2}/2$ is the mean radius of the spheroids. Only spheroids showing a regular spherical or quasi-spherical structure were analysed. Volume size determinations were carried out by two independent researchers to minimize possible subjective interference with the observed phenomenon. At the asymptotic volume levels error in the measurement procedure was estimated to be < 10% of the MTS volumes.

Standard deviation (SD) was estimated from repeat measurements of randomly selected volume samples. The greatest SD observed is shown in the figures.

Mathematical analysis of growth curves

Time-series of volume size determinations were fitted with the Gompertz growth equation (equation 1). Fitting was performed using two different algorithms for nonlinear least-square minimization: the Marquardt–Levenberg algorithm implemented in SigmaPlot (Jandel Scientific, Erkrath, Germany) and the 'fmins' function, which uses the Nelder–Mead simplex method, implemented in MatLab v.5.2 (MathWorks Inc., Natick, MA, USA). Standard statistical quantities were considered to best fit experimental data (e.g. r^2, SD and CV of estimated parameters). Residuals, i.e. experimental data minus calculated values by fitting, were analysed by Fast Fourier Transform and power spectrum analysis by taking advantage of algorithms implemented in MatLab. Mathematical models were also implemented in MatLab.

RESULTS

Analysis of the growth curves of individual MTS

Growth variability and saturation are observed *in vitro* when the growth of individual MTS is followed over a sufficiently long time-period (Fig. 1a). In time different MTS appeared to reach different asymptotic volumes following heterogeneous kinetics. In addition to the variability in the growth kinetics as defined above, a new type of variability can be appreciated in all analysed growth curves (see, e.g. Figure 1a) which consist of time-dependent fluctuations in the volume size of one MTS. At later times, the MTS volume fluctuates around the asymptotic volume predicted by the Gompertz growth model. The experimental and biological significance of the observed fluctuations have been addressed in a previous study (Chignola *et al*., 1999).

Best estimates of Gompertzian parameters V(0) α and β were used to calculate the instantaneous growth rate α_0 for each MTS (Brunton & Wheldon, 1980; Demicheli *et al*., 1989; Chignola *et al*., 1995). The instantaneous growth rate is the growth rate of a spheroid when it was ideally composed of one single cell, and it can be calculated using the following formula (Brunton & Wheldon, 1980; Demicheli *et al*., 1989; Chignola *et al*., 1995):

$\alpha_0 = \alpha + \beta \ln(V(0)/V_0)$, where α, β and V(0) have already been defined, and V_0 is the volume of one cell (assumed to be 10^{-6} mm^3; Brunton & Wheldon, 1980; Demicheli *et al*., 1989; Chignola *et al*., 1995). In essence, the instantaneous growth rate is a normalized parameter which allows one to compare the growth of different tumours. A strong linear correlation was found between α_0 and β for all MTS within the 9L and U118 populations (Fig. 1b), confirming findings reported previously (Chignola *et al*., 1995). Thus, the analysed MTS populations displayed the main growth characteristics shown by solid tumours in animals: (1) growth variability (2) growth saturation with variable saturation levels and (3) a strong linear correlation between α_0 and β.

Mathematical modelling of MTS growth

Growth curves were fitted with the Gompertz growth equation. If a model equation satisfactorily describes a certain process, then the residuals, i.e. the difference between observed and calculated data, should distribute randomly. A random distribution can be demonstrated by power spectrum analysis which reveals the frequency content of a time-series. In the case of a random distribution all frequencies have the same probability and the power spectrum is flat (white noise). As described previously (Chignola *et al*., 1999), power

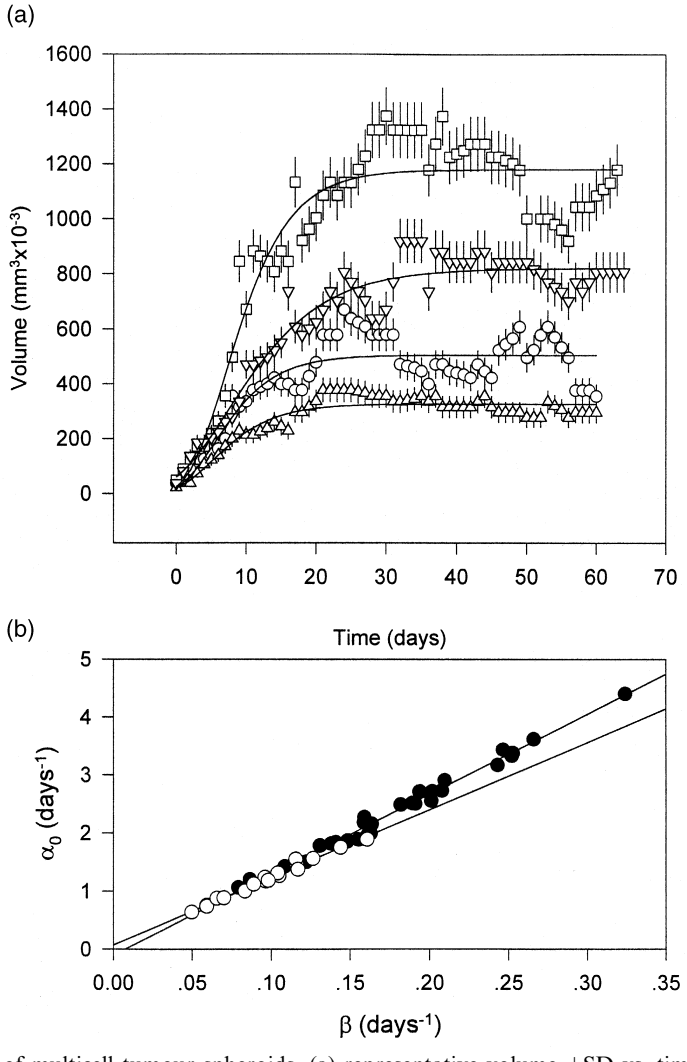

Figure 1. Growth of multicell tumour spheroids. (a) representative volume ±SD vs. time trajectories of four 9L MTS (○, □, △, ▽) fitted with the Gompertz growth equation (solid line). (b) the correlation found between the Gompertzian growth parameter a_0 and β is shown. Parameters were estimated by fitting of the individual growth curves observed for 31 9L MTS (●) and 18 U118 MTS (○).

spectra of the residuals computed for the growth trajectories of individual MTS fitted with the Gompertz growth equation were not flat. Plots of power spectra in a log-log scale revealed the existence of a correlation between frequency and amplitude (Fig. 2). This correlation is described by a power-law of the type $1/f^{\delta}$(pink noise) with a mean ± SD δ of 1.07 ± 0.25 and 1.26 ± 0.37 for 31 MTS obtained with 9L cells and 18 MTS obtained with U118 cells, respectively. Delta values measured for 9L MTS were compared with the values measured for U118 MTS using the *F*-test. The observed difference was statistically significant ($F = 4.2$, $P < 0.05$).

From a mathematical point of view, $1/f^{\delta}$spectra may be obtained by integration of white noise. Therefore, we developed a modified stochastic Gompertzian growth model. The

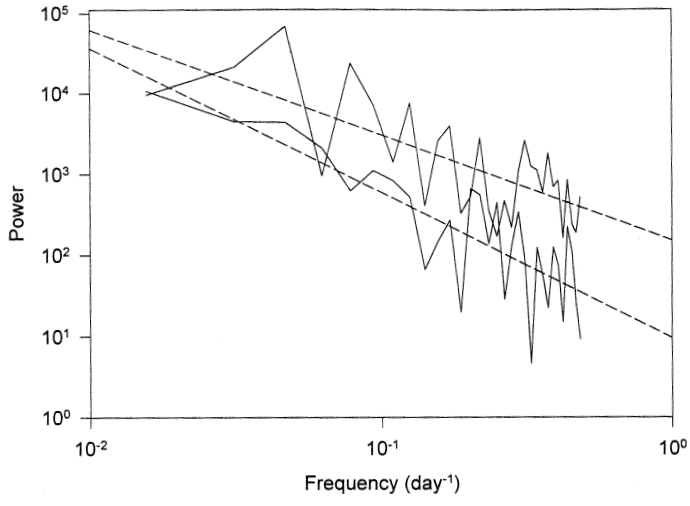

Figure 2. Power spectrum of the residuals computed for the individual growth trajectories of MTS. Two representative power-spectra are shown (continuous lines), along with the linear regression used to estimate the $1/f^{\delta}$ decay of the spectral amplitude with increasing frequency (dashed lines).

Gompertz equation shown above in analytic form (equation 1) is the exact solution of the following system of two differential equations:

$$\begin{cases} \dfrac{dV}{dt} = aV \\ \dfrac{da}{dt} = -\beta a \end{cases} \tag{3}$$

where α is a variable whose initial value coincides with α in equation 1. This system was modified according to:

$$\begin{cases} \dfrac{dV}{dt} = aV + w(t) \\ \dfrac{da}{dt} = -\beta a \end{cases} \tag{4}$$

where w(t) is a stochastic variable whose values are taken at random from a normal distribution with mean zero and variance λ^2. Equation 4 provides growth trajectories that depend on the initial conditions chosen for the variables V(0) and a(0) and for the parameter β and on the particular unpredictable realizations of the added white noise. Both initial conditions and β values can be estimated from experimental data. However, the proposed Gompertzian-like growth model (equation 4) would be hardly verified in its present form due to uncertainties in the variable w(t). We therefore modified equation 4 using the usual approximation:

$$\begin{aligned} \frac{dV}{dt} &\cong V(n+1) - V(n) \\ \frac{da}{dt} &\cong a(n+1) - a(n) \end{aligned} \tag{5}$$

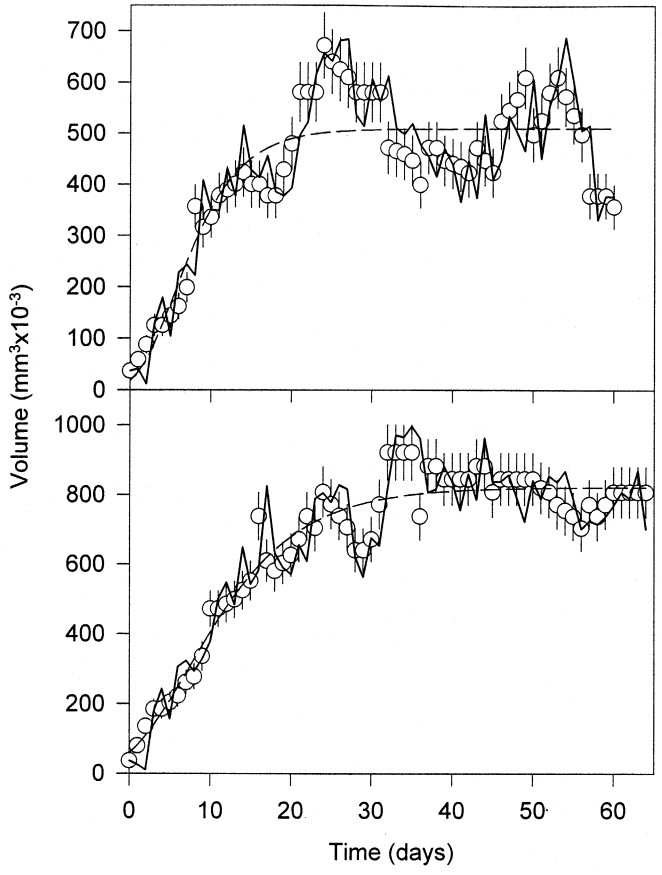

Figure 3. Forecasting the growth of MTS. Experimental data ± SD of volume size increase over time of two independent MTS. One-step prediction in the future of the volume dimensions was computed for each spheroid using equation 6 (continuous lines). For comparison, the classical Gompertzian trend fitted to experimental data is also shown (dashed lines).

that, combined with equation 4, results in the following model:

$$\begin{cases} V(n+1) = a(n)V(n) + w(n) + V(n) \\ a(n+1) = -\beta a(n) + a(n) \end{cases} \quad (6)$$

Model (6) was used to predict the volume of an MTS at time t(n + 1) starting from the volume measured experimentally at time t(n). For this purpose, the variance λ^2 of the added white noise was calculated from experimental data as follows:

$$\lambda^2 = \mathrm{var}\{[V(n+1) - V(n)]_{obs} - [V(n+1) - V(n)]_{calc}\}$$

where the subscripts 'obs' and 'calc' refer to MTS volumes measured experimentally or calculated by fitting with the Gompertz equation (equation 1), respectively.

One step predictions in the future of the volume of MTS are shown in Fig. 3. Residuals computed with respect to calculated data with equation 6 were randomly distributed. A random distribution was demonstrated by power spectrum of the residuals followed by statistical analysis using three different tests (Brockwell & Davis, 1996): Anderson,

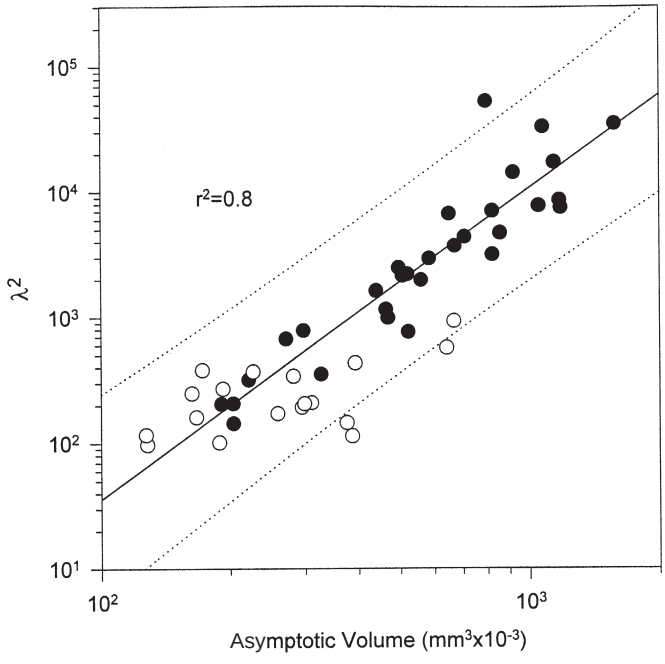

Figure 4. Correlation between noise intensity and asymptotic volume for the growth of MTS. Each point in the figure represents the value of the variance λ^2 plotted with respect to the asymptotic volume calculated for each spheroid within the 9L (●) and U118 (○) MTS populations. Prediction intervals at 99% level were also drawn in the figure (dotted lines).

Portmanteau and Cumulative Periodogram. Notably, our model predicts nicely either the nonstationary regimen (i.e. exponential-like growth at earlier times) and the quasi-stationary regimen (i.e. growth saturation at later times) of our experimental growth curves.

Growth variability and saturation in MTS

Results shown above demonstrate that both variability and saturation of MTS growth are best described by model 6. In our model, the source of variability is the added white noise whose intensity is given by the variance λ^2. A correlation was found between λ^2 and the asymptotic volume calculated for each of the individual MTS growth trajectories using equation 2 (Fig. 4). The prediction error was $< 10\%$ over 49 independent growth curves. No correlation was instead found when other Gompertzian parameters, i.e. α, α_0 and β were taken into account.

DISCUSSION

Mathematical analysis of the growth trajectories displayed by individual MTS revealed quantifiable growth variability and saturation typical of malignant proliferation. Growth variability is measured by means of power-laws of the type $1/f^{\delta}$ in power spectra and is additively superimposed to the S-shaped Gompertzian growth.

Power-laws are quite common in nature, and suggest the existence of scale-free internal dynamics, possibly chaotic, driving the behaviour of a system. Chaotic dynamics (e.g. deterministic variability) have been recently showed for the micromotion of tumour cells in

two-dimensional cultures (Posadas, Criley & Coffey, 1996). However, $1/f^{\delta}$ power spectra alone can not unambiguously discriminate between deterministic or stochastic dynamics. In fact, $1/f^{\delta}$ power spectra with small δ (i.e. less than 2) are also a result obtained when the dynamics of stochastic nonchaotic connected networks are analysed (Milotti, 1995).

Although the results shown in Fig. 2 did not allow us to reach a conclusion on the nature of MTS growth variability (i.e. stochastic vs. deterministic), they demonstrate that a source of variability must be considered to best model and forecast the growth of individual MTS. In principle, the source of growth variability might be present in the environment (i.e. environmental noise) or, intringuingly, the variability might be an intrinsic property of MTS owing to their three-dimensional organization (i.e. internal noise). Discriminating between environmental noise and internal noise may be important since, as shown in Fig. 4, the growth variability allows one to predict the course of MTS growth. The following reasons would suggest that the growth of individual MTS is driven by internal noise:

1 MTS were grown under the same controlled culture conditions (see Materials and methods). Thus, each MTS was subjected to the same environmental noise. The only perturbations applied to culture plates were limited to short periodic shifts from 37 °C to room temperature to carry out volume determinations and to change the growth medium. In previous work, we further limited external perturbations by changing medium only once during the whole growth assay for a control population of 9L MTS (Chignola *et al.*, 1999). However, the same type of growth variability was observed also for these MTS;

2 in spite of the variability of the observed asymptotic volumes, a strong linear correlation was found between parameters α_0 and β for all spheroids within the two analysed MTS populations (see Fig. 1). If the noise were environmental, then the growth of each spheroid would be perturbed in a less organized way. Under these conditions a linear correlation between growth parameters of independent growth curves would be hardly measured;

3 the correlation found between the variance (i.e. the intensity) of the added noise in our model and the asymptotic volume calculated for each MTS further support the conclusion that MTS growth is driven by internal noise.

It should be mentioned that, in physical terms, white noise corresponds to the behaviour of a dynamical process with n degrees of freedom (i.e. dynamics of n noninteracting variables). On the other side, coloured $1/f^{\delta}$ noise describes the behaviour of a dynamical system of interacting variables. In this case the degrees of freedom are reduced with respect to white noise dynamics and the system is more ordered.

In biological terms, the internal noise in spheroids might be interpreted as an internal energy of proliferation determining the time-evolution of MTS growth. The difference in calculated δ values between 9L and U118 MTS would suggest that this internal energy of proliferation may vary depending on the cell type. In traditional two-dimensional cell cultures, tumour cells are allowed to proliferate with, at least in principle, no constraints (i.e. as n noninteracting variables). In spite of this, chaotic-like oscillations were observed to occur for the micromotion of cultured tumour cells (Posadas *et al.*, 1996), that is to say a certain degree of order arises even under the less stringent experimental conditions of tumour cell proliferation. The increase of MTS volume is primarily due to the increase in cell number, though we cannot exclude other mechanisms like swelling of the spheroid structure. In MTS a strong constraint is imposed on cells since cells must grow in close contact to each other in order to form a three-dimensional aggregate. This would result in a decrease of the degrees of freedom of the proliferating cell population and might explain the observed $1/f^{\delta}$ power spectra. In other words, in MTS the proliferating cell population would constitute a

connected network of cells, each with a certain proliferation potential. The overall growth process would be the result of the dynamical balance between proliferation forces and cohesive forces acting on opposite directions over a great number of variables (i.e. cells). This interpretation might explain the observed fluctuating quasi-periodic growth pattern shown by MTS (Chignola *et al*., 1999).

Previous work showed that treatment of spheroids with antigen/receptor-specific cytotoxic protein molecules (immunotoxins) resulted in a heterogeneous effect: at certain immunotoxins concentrations some spheroids were sterilized (i.e. they did not grow after treatment) whereas others regrew after a variable delay time (Chignola *et al*., 1995). The therapeutic effect measured by applying the Gompertz growth model as the number of cells killed (log-kill) ranged between 0 and 4. This 4 logs range overlaps the range observed for the intensity of growth variability of MTS (see variance λ^2 in Fig. 4). It is therefore tempting to speculate that the heterogeneous effect of immunotoxin treatment and the variability in the growth dynamics of MTS could be correlated. However, this finding would require further investigation.

Overall, our data reveal the complex nature of the growth of three-dimensional tumour cell aggregates. MTS appear to possess the complexity shown by self-organized dynamical systems (Coffey, 1998), where the environmental noise as well as the internal noise play a fundamental role in regulating the behaviour of the system itself (Wiesenfeld & Moss, 1995).

For experimental tumours *in vivo* endpoints of growth process are hardly achieved during the observation period. Forecasting the endpoint of tumour growth might be of help in evaluating the effects of therapeutic regimens, e.g. by comparing endpoints of treated vs. nontreated tumours. Besides the biological and biophysical implications of the present work, these results suggest a way of measuring tumour growth potential *in vivo* by analysing the size variability in time of the tumour burden. The variability parameter λ^2 appears to predict the asymptotic volume of 3D-tumour cell aggregates better than it has been shown so far using traditional growth models.

ACKNOWLEDGEMENTS

The expert technical help of Mrs. T. Cestari is acknowledged. This work was supported in part by grants from the Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MURST 60%).

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