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Oscillating growth patterns of multicellular tumour spheroids

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Abstract. The growth kinetics of 9L (rat glioblastoma cell line) and U118 (human glioblastoma cell line) multicellular tumour spheroids (MTS) have been investigated by non-linear least square fitting of individual growth curves with the Gompertz growth equation and power spectrum analysis of residuals Residuals were not randomly distributed around calculated growth trajectories At least one main frequency was found for all analysed MTS growth curves, demonstrating the existence of time-dependent periodic fluctuations of MTS volume dimensions Similar periodic oscillations of MTS volume dimensions were also observed for MTS generated using cloned 9L cells However, we found significant differences in the growth kinetics of MTS obtained with cloned cells if compared to the growth kinetics of MTS obtained with polyclonal cells Our findings demonstrate that the growth patterns of three-dimensional tumour cell cultures are more complex than has been previously predicted using traditional continuous growth models

INTRODUCTION

Multicellular tumour spheroids (MTS) are three-dimensional aggregates of tumour cells that can be obtained and cultured *in vitro* under controlled experimental conditions (Sutherland 1988) MTS represent a tumour model with an intermediate complexity between standard two-dimensional monolayer cultures *in vitro* and tumours *in vivo*, as they approximate many biological characteristics of micrometastases or of intervascular regions of larger tumours (Sutherland 1988) Among these, MTS show (1) heterogeneous expression of cell surface molecules, (2) production of an intercellular matrix, (3) heterogeneous distribution of nutrients and of cell proliferation, (4) presence of a central core of quiescent and eventually necrotic cells (Sutherland 1988) Owing to their biological properties, MTS are amenable for studying the therapeutic efficacy of anti-cancer therapies under more realistic conditions than those obtainable with traditional two-dimensional cell cultures Major differences in sensitivity to drugs have been demonstrated in tumour cells grown as spheroids due to a limited diffusion and uptake of therapeutic molecules and/or to microenvironmental factors originated by the three-dimensional architecture (Twentyman 1980, Sutherland 1988, Rofstad & Sutherland 1989, Steeg *et al* 1994, Chignola *et al* 1994)

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The growth kinetics of MTS parallel those of *in vivo* tumours, and they have been found to obey the Gompertz growth model (Demicheli *et al.* 1989). Several biological and theoretical findings have been derived indicating that the Gompertz model is the best descriptor of biological growth in general and of tumours and MTS growth in particular (Laird 1964, Laird 1969, Xu 1987, Demicheli *et al.* 1989, Ling & He 1993). Moreover, analysis of tumour growth with the Gompertz equation allows one to quantify the outcome of anti-tumour therapies (Lloyd 1975, Bassukas 1993, Chignola *et al* 1995) and to predict the course of tumour progression *in vivo* even from a few early determinations of tumour size (Norton *et al.* 1976).

Understanding the growth of tumours may have profound implications in experimental and clinical oncology. Experimental time series of tumour size measurements can be fitted with growth equations. A few determinations of tumour size are in general considered sufficient to provide a best fit to experimental data with non-linear equations. However, new information on the biology of tumour growth might be gathered from more detailed kinetic assays where measurement of tumour size are sampled accurately. Increasing the number of tumour volume determinations *in vivo* may be hampered by technical limitations. This is not the case for MTS *in vitro* where the time-dependent variations of the volume dimension can be measured with great precision.

Accurate determinations of MTS volume in kinetic assays followed by data analysis using signal-analysis techniques revealed the existence of new tumour growth patterns. These patterns consist of temporal periodic fluctuations of MTS size and were not predicted by the traditional Gompertz growth model or by related continuous growth models.

The volume of a spheroid is a measure of the size of the tumour cell population forming a three-dimensional aggregate. MTS are likely to be generated by aggregation of a few cells heterogeneous with respect to their proliferation kinetics. To further investigate whether the observed periodic oscillations in time of MTS volume size could be related to the proliferating effects of heterogeneous cell subpopulations, MTS were also generated using either cloned cells (mMTS) or polyclonal cells (pMTS).

MATERIALS AND METHODS

Generation of multicellular tumour spheroids

Basic method

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.

Monoclonal and policlonal spheroids

9L cells were cloned by seeding 0 1–0 3 cells/well into the wells of a 96-well culture plate After 17 days, 19 out of 96 wells reached a confluent growth (95% plating efficiency) Since under these conditions one well contains approximately 20 000 cells we estimated (assuming exponential growth) a mean doubling time $T_d \approx 28$ h for this cloned cell population, a value close to the mean doubling time of the 9L cell population estimated to be $T_d \approx 24$ h Cloned adherent cells were removed by trypsin wash and placed into the wells of a 24-well culture plate on a layer of a 0 75% (w/v) of agar in RPMI-FBS to form spheroids Spheroids were then processed as described above We will refer to this spheroid population as monoclonal MTS (mMTS) As a control, 10^4 cells were seeded into the wells of a 96-well culture plate After 24 h, cells were processed in parallel as described for the cloned MTS population We will refer to this spheroid population as polyclonal MTS (pMTS) In growth assays with mMTS and pMTS old medium was replaced by fresh medium only once on the 33rd day In doing so we aimed at minimizing possible perturbations of MTS growth due to periodic changes of growth medium However, this experimental condition was not observed to limit the growth of either mMTS or pMTS

Mathematical analysis of growth curves

A time-series of volume size determinations were fitted with the Gompertz growth equation

$$V(t) = V_0 \exp\{\alpha/\beta [1 - \exp(-\beta t)]\}$$

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 Fitting was performed using two different algorithms for non-linear least-square minimization the Marquardt–Levenberg algorithm implemented in SigmaPlot (Jandel Scientific) and the 'fmins' function, which uses the Nelder–Mead simplex method, implemented in MatLab v 5 2 (MathWorks, Inc , Natick, MA) 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

Statistics

Under the culture conditions described above, each spheroid within an MTS population (e g mMTS) grows independently of all other spheroids of the same population A certain degree of variability in the growth parameters estimated for MTS belonging to one MTS population is therefore expected To take into consideration the intra- and the inter-population growth variability, comparisons between growth parameters of mMTS and pMTS populations were carried out by variance analysis using the F-test A F-value above the 0 05 probability level was considered not significant

RESULTS

Analysis of MTS growth

The growth of MTS was analysed by generating 24 spheroids with 9L cells and 19 spheroids with U118 cells Each volume time-series was fitted with the Gompertz growth equation (Figure 1, top panel) Residuals were then computed for each series within these MTS populations (Figure 1, middle panel)

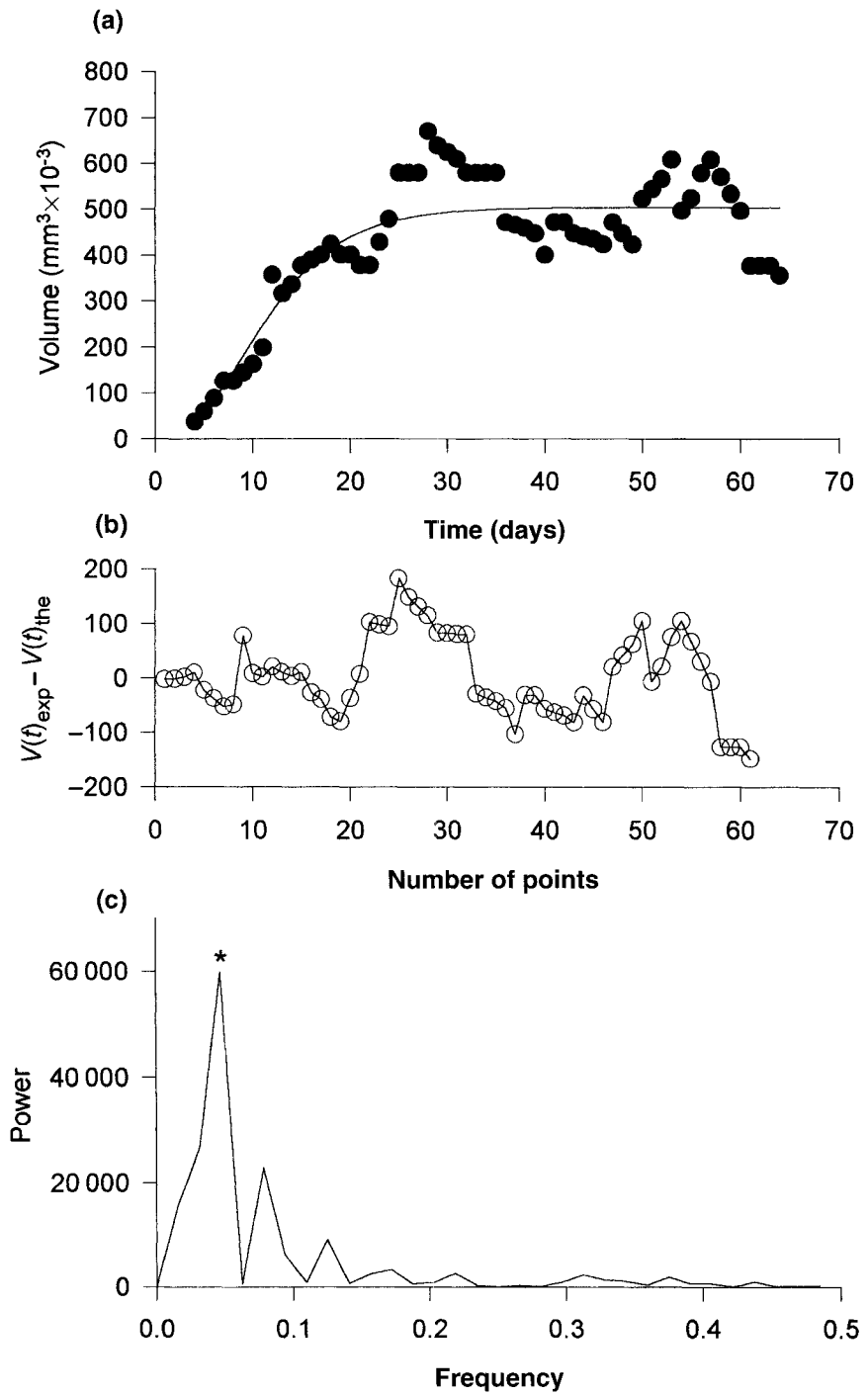

Figure 1. Growth of MTS.A representative experiment is shown. (a) Raw data of volume size vs. time measured for one 9L MTS (symbols) and best fitting with the Gompertz equation (continuous line). (b) Plot of the residuals calculated for the data shown in the top panel. (c) Power spectrum of the residuals. The main peak is indicated by an asterisk.

If spheroid growth were fully described by the Gompertz growth model, then a random distribution of residuals around calculated growth curves should be expected 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 the spectrum is flat with a constant power at all frequencies When the residuals obtained by fitting of MTS growth with the Gompertz equation were subjected to Fast Fourier Transform (FFT) and power spectrum analysis, at least one main peak in the power spectrum was observed for the growth of each of the 24 9L spheroids and of each of the 19 U118 spheroids (Figure 1, bottom panel) The observed frequency of the main peak varied in the range 0 0156–0 0938 day^{-1} and 0 0312–0 1562 day^{-1} for the residuals calculated for different 9L and U118 spheroids, respectively (Table 1)

A main peak in the power spectrum indicates the presence of an oscillatory pattern at the corresponding frequency value Residuals of different growth curves fluctuated with a different frequency No correlation was found between estimated frequency values and any of the estimated Gompertz growth parameters (Table 1) Thus, the observed power spectra demonstrate that (1) the volume size of MTS oscillates in time and that (2) this fluctuating pattern, if considered as additively superimposed, does not result to be dependent on the gompertzian growth

Table 1. Relationships between estimated Gompertz growth parameters and frequency of the main peak in power spectra for 9L and U118 MTS

MTS number	9L			U118		
	α (day $^{-1}$)	β (day $^{-1}$)	Frequency (day $^{-1}$)	α (day $^{-1}$)	β (day $^{-1}$)	Frequency (day $^{-1}$)
1	0 618	0 121	0 0469	0 462	0 116	0 0625
2	0 950	0 181	0 0461	3 036	0 249	0 0937
3	1 233	0 178	0 0156	0 260	0 059	0 0468
4	1 092	0 181	0 0938	0 571	0 105	0 0781
5	0 506	0 149	0 0625	1 053	0 115	0 0625
6	0 204	0 030	0 0625	0 595	0 160	0 0781
7	1 912	0 183	0 0781	0 191	0 030	0 0312
8	0 580	0 160	0 0156	0 210	0 059	0 0468
9	1 406	0 223	0 0156	0 357	0 096	0 0625
10	1 586	0 236	0 0156	0 359	0 065	0 1562
11	1 092	0 182	0 0234	0 197	0 049	0 0937
12	1 619	0 221	0 0938	0 384	0 104	0 0468
13	1 072	0 171	0 0156	0 224	0 083	0 0468
14	0 986	0 172	0 0469	0 286	0 117	0 0468
15	1 117	0 193	0 0156	0 382	0 096	0 0468
16	0 580	0 131	0 0323	0 309	0 098	0 0625
17	0 652	0 163	0 0313	0 294	0 070	0 0625
18	1 664	0 222	0 0156	0 382	0 126	0 0468
19	0 755	0 127	0 0469	0 370	0 088	0 0625
20	2 162	0 264	0 0469			
21	1 087	0 170	0 0859			
22	1 472	0 201	0 0156			
23	1 431	0 171	0 0156			
24	0 928	0 143	0 0469			
mean	1 113	0 174	0 0410	0 522	0 099	0 0650
±SD	± 0 48	± 0 046	± 0 026	± 0 64	± 0 047	± 0 027

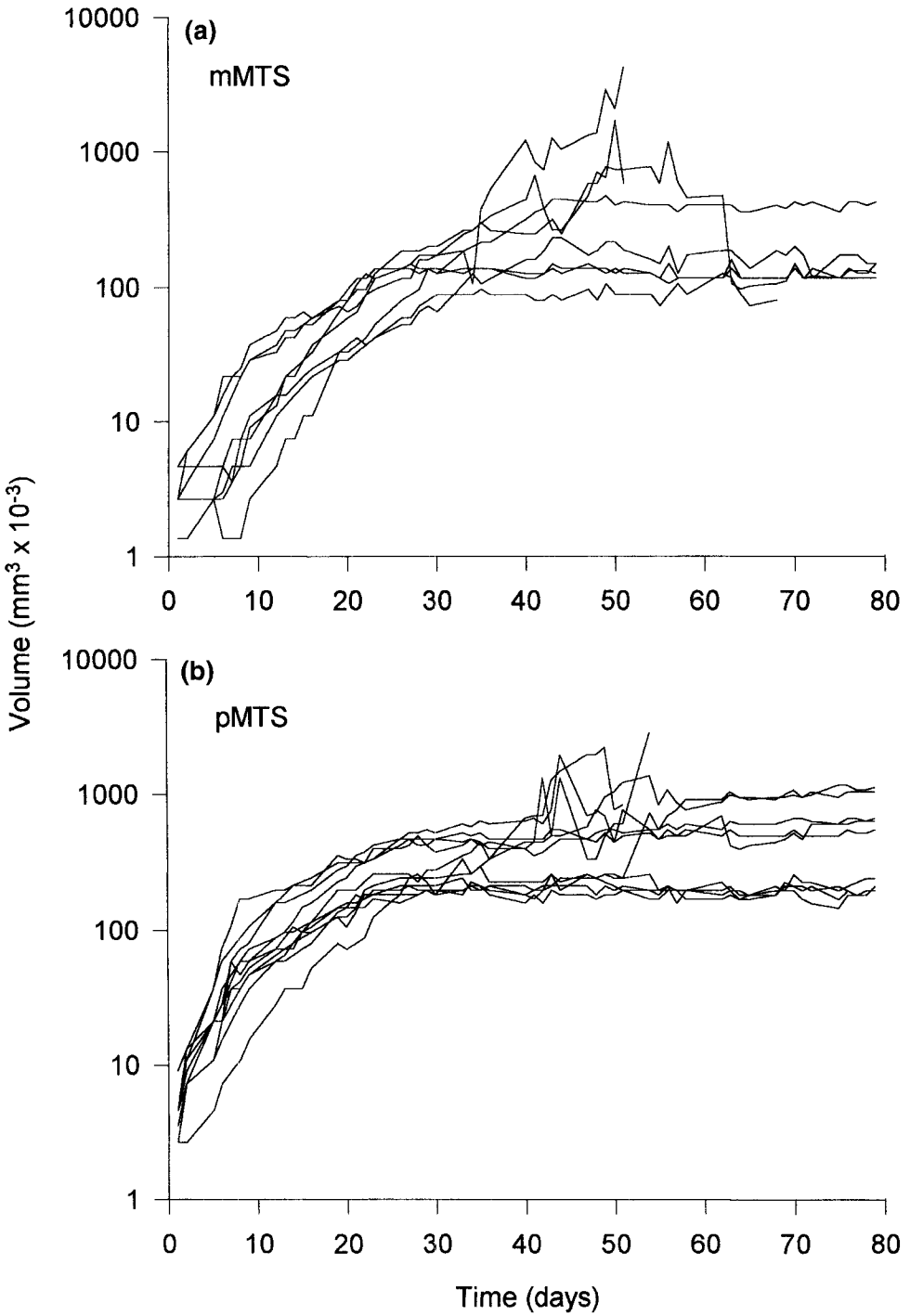

Figure 2. Observed volume vs. time trajectories of mMTS (a) and of pMTS (b).

Growth of MTS generated using cloned or polyclonal 9L cells

The growth trajectories of 8 mMTS and of 11 pMTS were analysed (Figure 2) Fitting with the Gompertz equation and analysis of residuals by FFT and power spectrum revealed the presence of a main peak in the power spectra, and hence of periodic oscillations in time of volume size, for series belonging to either mMTS and pMTS populations No clear differences between mMTS and pMTS were observed in the frequency of the main peak in power spectra (Table 2)

Differences were instead observed in the growth kinetics of mMTS as compared to pMTS at early times in the growth curves as it can be appreciated in Figure 2 mMTS appear to grow more slowly than pMTS To further analyse the differences between mMTS and pMTS growth kinetics, we considered the transitions in time of the spheroid volume dimensions for each mMTS or pMTS A transition of 1 means that the volume size at time t_{n+1} increases by one unit in the used discrete metric scale with respect to the value measured at time t_n The relative frequency of the volume transitions measured for all mMTS and pMTS in the growth time-interval 0–15 days are plotted in Figure 3 The mode of this distribution is at a lower volume transitions for mMTS than for pMTS The overall mean number of volume transitions ≤ 1 measured for mMTS and pMTS was $7\,5 \pm 1\,77$ and $5\,09 \pm 1\,22$, respectively (Table 3) The total number of volume transitions ≤ 1 measured for each spheroid of the mMTS population was compared with the values measured for each spheroid of the pMTS population using the *F*-test The observed difference was statistically significant ($F = 12\,38$, $P < 0\,01$), indicating that mMTS grew more slowly than pMTS during the considered time-interval No statistically significant differences were observed when later growth time-intervals were considered

Table 2. Comparison between Gompertz growth parameters and frequency of the main peak in power spectra estimated for mMTS and pMTS

MTS number	α (day $^{-1}$)	β (day $^{-1}$)	Frequency (day^{-1})
mMTS			
1	1 619	0 174	0 0156
2	1 372	0 102	0 0234
3	0 678	0 097	0 0234
4	0 703	0 103	0 0391
5	0 193	0 021	0 0781
6	0 291	0 014	0 0625
7	0 874	0 082	0 0234
8	0 859	0 147	0 0547
Mean ± SD	0 824 ± 0 48	0 093 ± 0 05	0 040 ± 0 022
pMTS			
1	0 862	0 160	0 0156
2	0 658	0 084	0 0313
3	0 981	0 137	0 0391
4	0 847	0 146	0 0469
5	0 770	0 078	0 0313
6	0 458	0 056	0 0938
7	0 992	0 159	0 0469
8	0 321	0 038	0 0234
9	0 677	0 053	0 0781
10	0 513	0 111	0 0156
11	0 677	0 119	0 0391
Mean ± SD	0 705 ± 0 213	0 104 ± 0 044	0 0419 ± 0 024

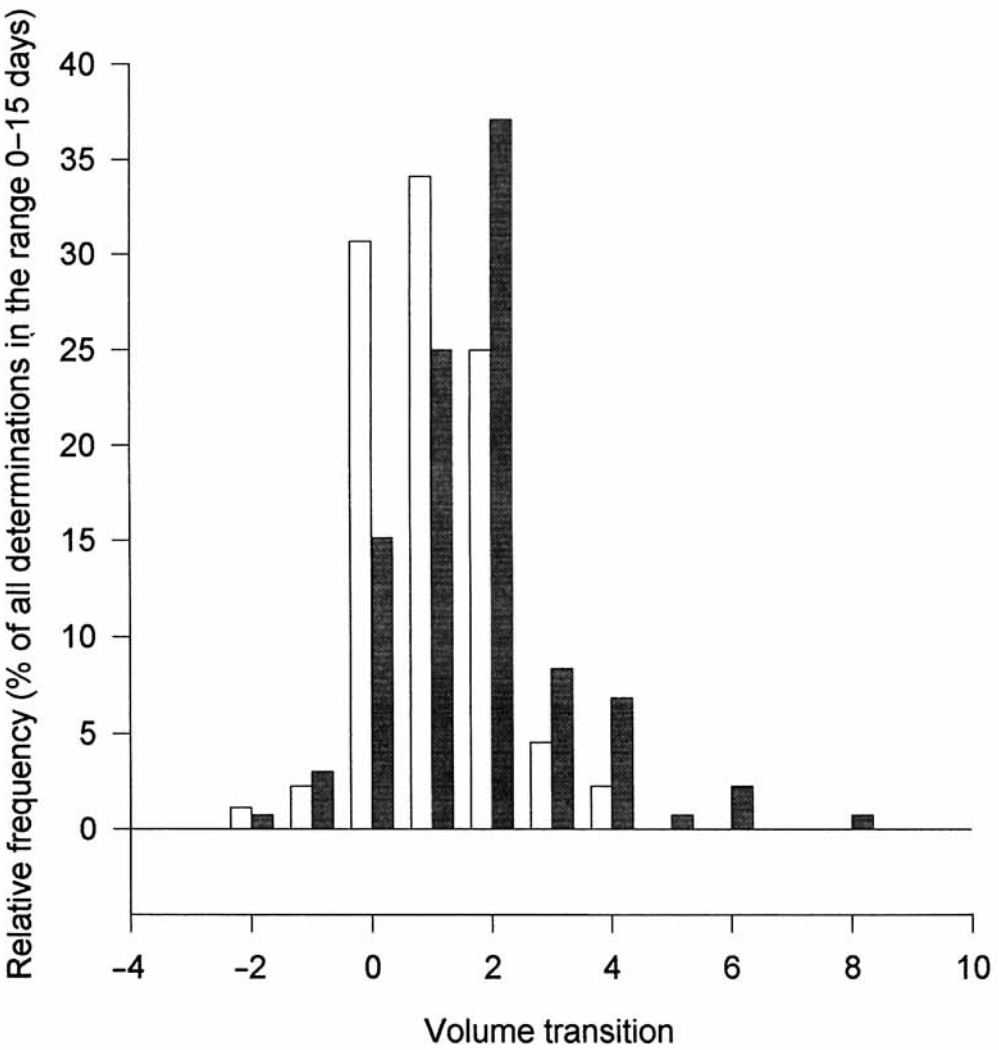

Figure 3. Plot of the distribution of the measured volume transitions in the time interval 0–15 days for mMTS (white bars) and pMTS (grey bars).

DISCUSSION

Detailed analysis of spheroid growth kinetics revealed the presence of time-dependent low-frequency oscillating patterns of the volume dimensions apparently superimposed on the assessed Gompertzian trend. We ruled out the possibility that these growth patterns could be the result of experimental artefacts for the following two reasons:

(1) volume size determinations were carried out by two independent researchers to minimize possible subjective interference with the observed phenomenon. Errors due to imprecision in spheroid volume determination would be expected to distribute randomly around experimental measures, and therefore would provide flat power spectra.;

Table 3. Number of volume transitions ≤1 measured for each spheroid within the mMTS and pMTS populations in the time-interval 0–15 days

MTS number	mMTS	pMTS
1	6	5
2	10	3
3	6	5
4	9	4
5	5	5
6	9	7
7	8	4
8	7	7
9		5
10		6
11		5
Total	60	56
Mean ± SD	7 5 ± 1 77	5 09 ± 1 22

(2) periodic variations in the culture conditions were limited to daily short temperature shifts from 37°C to room temperature to carry out measures of volume size under the light microscope Periodic shifts of culture plates from 37°C to room temperature were also made to change the growth medium However, (a) a one-day period oscillation was not observed in power spectra and (b) oscillating growth patterns were observed also for mMTS and pMTS spheroid populations where medium was changed only once during the whole growth time-period

Oscillations of the size of a biological population are observed in nature and also predicted by ecological mathematical models (e g Lotka–Volterra and related prey-predator models), and suggest the existence of some dynamical interactions among competing populations (Murray 1989) We aimed at exploring this possibility with experimental three-dimensional tumours by generating spheroids using cloned, hence homogeneous, 9L cells However, oscillating growth patterns were observed also for this spheroid population Thus time-dependent oscillation of volume dimensions appeared not to be related to a possible spheroid composition in heterogeneously growing cell subpopulations competing with each other Within a spheroid, cells can be found in different proliferation states actively proliferating in the outer rims, non-proliferating/quiescent in the inner layers (Sutherland 1988) Irrespective of the composition of MTS in polyclonal or monoclonal cells, the observed oscillatory growth might therefore be due to heterogeneity in the subpopulations of cycling (i e proliferating) versus non-cycling (i e non-proliferating/quiescent) cells

Differences in the growth kinetics were observed for mMTS with respect to pMTS At early times in the growth curve mMTS showed a significant slowing down of the growth process as compared to pMTS At later times, the growth kinetics of mMTS and of pMTS were not statistically different, with similar final asimptotic volume dimensions This finding would imply that a composition of MTS in tumour cell subpopulations heterogeneous with respect to the proliferation kinetics might confer a selective advantage resulting in initial faster kinetics of three-dimensional tumours

In conclusion, the oscillating growth displayed by 9L and U118 spheroids appears to be a real phenomenon due to some as yet unexplored biological and/or biophysical

growth properties of experimental tumour cell aggregates. Our findings should be taken into consideration in developing theoretical growth models of tumour micrometastases.

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