Gompertzian growth pattern correlated with phenotypic organization of colon carcinoma, malignant glioma and non-small cell lung carcinoma cell lines

M. A. A. Castro^{*,†}, F. Klamt^{*,†}, V. A. Grieneisen[‡], I. Grivicich[†]
and J. C. F. Moreira^{*}

^{*}Departamento de Bioquímica, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil,

[†]Universidade Luterana do Brasil, Canoas, RS, Brazil, and [‡]Instituto de Física, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

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Abstract. In the current study we present a Gompertzian model for cell growth as a function of cell phenotype using six human tumour cell lines (A-549, NCI-H596, NCI-H520, HT-29, SW-620 and U-251). Monolayer cells in exponential growth at various densities were quantified over a week by sulforhodamine B staining assay to produce cell-growth curves. A Gompertz equation was fitted to experimental data to obtain, for each cell line, three empirical growth parameters (initial cell density, cell-growth rate and carrying capacity – the maximal cell density). A cell-shape parameter named deformation coefficient D (a morphological relationship among spreading and confluent cells) was established and compared by regression analysis with the relative growth rate parameter K described by the Gompertz equation. We have found that coefficient D is directly proportional to the growth parameter K . The fit curve significantly matches the empirical data ($P < 0.05$), with a correlation coefficient of 0.9152. Therefore, a transformed Gompertzian growth function was obtained accordingly to D . The degree of correlation between the Gompertzian growth parameter and the coefficient D allows a new interpretation of the growth parameter K on the basis of morphological measurements of a set of tumour cell types, supporting the idea that cell-growth kinetics can be modulated by phenotypic organization of attached cells.

INTRODUCTION

Among the central questions in tumour biology is the extent of the role of cell phenotype in modulation of cell growth (Folkmann & Moscona 1978; Ingber 1990; Chen *et al.* 1997). In previous studies of three human non-small cell lung carcinoma cell lines (Castro *et al.* 1999; Castro, Schwartzmann & Moreira 2001), we addressed the question of whether two- and

three-dimensional growth followed a common growth dynamic. We have shown the use of morphometrical analysis to describe each cell line and concluded that the cells under study gave rise to phenotypic growth modulation as a function of cell density.

We also report here a phenotypic interpretation of the Gompertz model according to the growth pattern of six human tumour cell lines. This mathematical method may express a property of biological growth as a self-organized process modulated by cell phenotype.

The increase in cell number with time that characterizes many biological systems is often well described by mathematical growth curves. Among the classical models of tumour growth, the Gompertz model provides a satisfactory fit with growth data (Marusic *et al.* 1991; Marusic *et al.* 1994). The growth behaviour described by the Gompertz equation has an exponential nature at the early stage, after which it saturates, approaching a plateau as the tumour size increases. The essential distinguishing feature of Gompertzian growth is some mechanism of feedback inhibition with increasing cell number that achieves a limiting size asymptotically (Norton *et al.* 1976; Vinay & Alexandro 1982). It has been suggested that cell-shape compression of confluent cells may control the inhibitory feedback as a geometrical arrest of mitosis, reflecting the influence of the cytoskeletal network organization (Wang *et al.* 1993; Olive & Durand 1994). For example, Chen *et al.* (1997) have found that, as cells become rounded, DNA synthesis gradually stops. Inversely, most cells require spreading on extracellular matrix substrate for proper growth and normal function (Ruoslahti 1997). Such relationships among growth and shape have been studied in this paper by regression analysis of a cell deformation parameter, which describes the amplitude of cell shape variation, showing positive correlation with kinetic parameters of growth described by the Gompertz model.

MATERIALS AND METHODS

Cell culture

The human non-small cell lung carcinoma cell lines A-549, NCI-H596 and NCI-H520, the colon carcinoma cell lines SW-620 and HT-29 and the U-251 malignant glioma cell line were obtained from the American Type Culture Collection (Rockville, MD, USA). All cell lines were grown as an adherent monolayer in 25-cm² culture flasks at 37 °C in a 5% CO₂ humidified atmosphere and maintained in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% FCS (fetal calf serum; v/v). Trypsin treatment was carried out at 37 °C for 2 min with a mixture of 0.05% (w/v) trypsin and 0.02% (w/v) EDTA. After trypsinization, cells were counted in a haemocytometer chamber, diluted to appropriate numbers and seeded.

Cell growth curves

Cells were separated into a single-cell suspension in culture medium by trypsinization and seeded into 96-well culture plates at a density of 7.5×10^3 cells/cm² (initial cell density). Cell growth was performed at 37 °C in a 5% CO₂ humidified atmosphere for at least 1 week. The medium was replenished each 48 h after seeding and the cell number was determined periodically by the sulforhodamine B (SRB) staining assay as described elsewhere (Skehan *et al.* 1990). Briefly, adherent cell cultures were fixed *in situ* by adding 50 µl of cold 50% (w/v) trichloroacetic acid (TCA) (final concentration, 10% TCA) for 60 min at 4 °C. The supernatant was then discarded, and the plates were washed five times with deionized water and dried. One hundred microlitres of SRB solution (0.4% in 1% acetic acid, w/v) was added to each microtitre well and the culture was incubated for 10 min at room temperature (~25 °C). Unbound SRB was removed

by washing five times with 1% acetic acid and the plates were air dried. The bond stain was solubilized with Tris buffer, and absorbance was read on an automated spectrophotometric plate reader at a single wavelength of 515 nm. Cell growth was assessed in the SRB-staining assay by whole culture protein determination, showing sensitivity and reproducibility. Because of its large linearity range, the SRB assay is suitable to study, not only low-density cultures, but also subconfluent monolayers and multilayer cell clusters containing large amounts of cells (Keepers *et al.* 1991; Pizao *et al.* 1992).

Best-fit curves and non-linear regression analysis

Tumour cell growth was characterized according to the Gompertz model by fitting the Gompertz equation to experimental data for each individual cell line (Norton *et al.* 1976; Vinay & Alexandro 1982). At its conception (Gompertz 1825), this model was used to deal with human mortality and, unexpectedly, it was later found useful to describe biological growth (for model review see Bajzer & Vuk-Pavlovic 1996). A Gompertzian function is the solution of the differential equation

$$dn/dt = -K \cdot n \cdot \ln(n/\theta) \quad (1)$$

where K is a constant > 0 , n is the cell number at t time and θ is the carrying capacity (the maximal cell density). Biologically, the growth fraction can be interpreted as $\ln(n/\theta) = g(n)$, i.e. the ratio of proliferating cells versus total cell population. This concept requires that $g(n) \leq 1$ and that parameter K be interpreted as the growth-rate constant. Consequently, cells reach the carrying capacity only asymptotically. The solution of the equation 1 is often written in the form

$$n(t) = \theta \cdot \exp[-\ln(\theta/n_0) \cdot \exp(-K \cdot t)] \quad (2)$$

For each tumour cell line, the solution of the Gompertz differential equation was fitted using the least square method to experimental data of cell density as a function of time of growth, starting from 7500 cells/cm². The growth parameters were obtained using the SPSS (Chicago, Illinois, USA) non-linear regression package with Marquardt's method, which combines the best features of the Gauss and Steepest descent method (Vinay & Alexandro 1982).

Cell-shape measurements and statistical analysis

Morphometrical measurements were obtained by NIH-image program analyses of the scanned phase contrast photomicrographs of cells plated as dispersed and confluent densities. At least 30 cells from three experiments were measured to estimate shape parameters of each cell line. The correlation among continuous variables was analysed by Pearson correlation coefficient. Data are reported as mean $\pm$ SD, with the level of significance set at $P < 0.05$.

RESULTS

The cell-shape polarity of attached cells is described in this paper by the relationship among two orthogonal cell diameters, illustrated in Fig. 1 by comparing morphologically distinct cells. The ratio of the larger diameter r_i and the smallest diameter r_j gives the cell polarity

$$f = r_i/r_j \quad (3)$$

Figure 1. Illustration procedure for the SW-620 cell line to estimate the deformation coefficient D . Phase contrast photomicrographs were analysed in NIH-image program and two orthogonal cell diameters (r_i , r_j) were measured in both spread (a) and confluent (b) cell culture phenotypes. The cell polarity f is expressed by the ratio $f = r_i/r_j$. Coefficient D was obtained by the formula $D = f_0/f_1 - 1$, where f_0 is the cell polarity of spread cells and f_1 the polarity of confluent cells. A cell foci with heterogeneous cells in shape is shown in (c) and exemplifies the amplitude of cell distortion expressed by the cell deformation coefficient D . At least 30 cells of each line in three experiments were measured to obtain the mean $\pm$ SD presented in Table 1 for A-549, NCI-H596, NCI-H520, HT-29, SW-620 and U-251 cell lines. Bar in (c), 50 μ m.

Table 1. Cell shape and growth parameters

Cell line	Polarity ^a f_0	Polarity ^a f_1	Deformation ^b coefficient D	Growth ^c constant K
A-549	3.72 ± 0.19	1.96 ± 0.17	0.90 ± 0.22	0.68 ± 0.14
NCI-H596	3.10 ± 0.34	2.02 ± 0.21	0.53 ± 0.16	0.47 ± 0.08
NCI-H520	1.82 ± 0.08	1.37 ± 0.09	0.33 ± 0.05	0.32 ± 0.09
HT-29	2.33 ± 0.61	1.40 ± 0.23	0.67 ± 0.12	0.76 ± 0.18
SW-620	2.63 ± 0.92	1.30 ± 0.20	1.03 ± 0.17	0.91 ± 0.26
U-251	4.54 ± 1.92	3.55 ± 1.12	0.32 ± 0.06	0.38 ± 0.16

^{ab}Mean $\pm$ standard deviations are given. No less than 30 cell outlines from three experiments were used to estimate morphological parameters in each cell group. ^aCell polarity at sparse (f_0) and confluent (f_1) conditions. ^bCell deformation coefficient obtained according to equation 4: $D = f_0/f_1 - 1$. ^cGrowth parameter obtained by fitting the Gompertz equation 2 to cell growth data of the Fig. 2. The parameter K is presented as the mean $\pm$ SEM of three different growth data for each cell line.

a parameter ≥ 1 (i.e. for round cells $f = 1$). The amplitude of the polarity variation is expressed by the coefficient $D \geq 0$

$$D = f_0/f_1 - 1 \tag{4}$$

where f_0 is the polarity of cells at sparse culture and f_1 the polarity of confluent cells. Therefore, the parameter D can be understood as a morphological deformation coefficient of attached cells. Similar cell measurements have been done previously to characterize functional heterogeneity of attached cells (Thoumine *et al.* 1995; Digina *et al.* 1998). The shape parameters obtained from our tumour cell panel are presented in Table 1.

Figure 2. Growth curves of monolayer cells. The solid lines are the best-fit curves of the Gompertz equation 2 and the points are experimental data of our cell panel: SW-620 (○), HT-29 (□), U-251 (△), NCI-H520 (●), NCI-H596 (■) and A-549 (▲) cell lines. The cell-density scale is transformed logarithmically. The growth-rate constant K derived from the best-fit curves of each cell line is given in Table 1 and is plotted in Figure 3 along with the deformation coefficient as paired constants (K, D). Each symbol represents the average value of at least three experiments. Standard deviations were typically 10% of the mean for cell number.

In order to compare D with kinetic parameters of cell growth, the cell lines were seeded at 7500 cells/cm² and incubated for at least 1 week to produce the growth curves presented in Fig. 2. For each tumour cell line, the solution of the Gompertz differential equation (equation 2) was fitted to experimental growth data. The best-fit curves match closely the actual data observed in the growth of the tumour cells. The constant K derived from several such curve-fits is presented in Table 1 and is described as a growth rate parameter, accordingly to the Gompertz model. Assuming the linearity for fitting procedures, when K is plotted against D in Fig. 3 for each cell line, the linear relationship exhibits a correlation coefficient of 0.9152 ($P < 0.05$). This degree of correlation between K and D allows, for these tumours cells, a new interpretation of K on the basis of an estimate of D produced from morphological measurements. Equation 2 may therefore be written

$$n(t) = \theta \cdot \exp[-\ln(\theta/n_0) \cdot \exp(-(D - m) \cdot t/u)] \quad (5)$$

a Gompertzian equation with a phenotypic variable, D , with constants u and m derived from the regression equation $D = u \cdot K + m$. Although a six-cell-line panel produces a limited set of the data points to assume the linearity among K and D for a different tumour growth model, i.e. suspension cell cultures and multi-cell spheroids (Olive & Durand 1994), our finding may be representative for tumour cell lines established as monolayers. Therefore, the assumption in equation 5 states that the measurable cell number $n(t)$ changes with t as described in the Gompertz modal (equation 2), but with a phenotypic growth rate. We present in Fig. 4 the simulation of cell growth using equation 5 instead of the equation 2. The best-fit curves – as shown in Fig. 2 – are also plotted in Fig. 4 to observe the results of both procedures. The growth rate was

Figure 3. Correlation between growth rate parameter K and deformation coefficient D . The points represent pairs of (K, D) determined from morphological measures as illustrated in Figure 1 and from the best-fit curves presented in Figure 2 for U-251 (a), NCI-H520 (b), NCI-H596 (c), HT-29 (d), A-549 (e) and SW-620 (f) cell lines. The whole data set are given in Table 1, including the confidence intervals (error bars) for each parameter. The Pearson correlation coefficient of the regression equation $D = u \cdot K + m$ is present inside the box. The u and m -values are empirically determined. Correlation is significant at the level of $P < 0.05$.

Figure 4. Model prediction of the growth curves. The transformed Gompertzian equation 5 was used to simulate cell growth (solid lines) of the SW-620 (a), HT-29 (b), U-251 (c), NCI-H520 (d), NCI-H596 (e), and A-549 (f) cell lines. Simulations were produced with the deformation coefficient D of each cell line presented in Table 1 and with the constants $u + m$ of the Figure 3, in agreement with the description of equation 5. The best-fit curves of experimental data (dotted lines) are the same as presented in Figure 2.

the only difference established among the equations 2 and 5 for each cell line, given that K is replaced with the coefficient D (together with $u + m$ constants) as described in equation 5.

In addition, the conclusion that $K \propto D$ can be theoretically derived by making two assumptions. If

$$f(n) = f(\theta) + [(f(n_1) - f(\theta))/\ln(n_1/\theta)] \cdot \ln(\theta/n) \quad (6)$$

where $n_1 < \theta$ and $f(n) = r_i(n)/r_j(n)$, and

$$\alpha(n) \propto [(f(n) - f(\theta))/f(\theta)] \cdot \ln(\theta/n_1) \quad (7)$$

then if growth is Gompertzian, given by

$$dn/dt = \alpha(n) \cdot n \quad (8)$$

where

$$\alpha(n) = K \cdot \ln(\theta/n) \quad (9)$$

then substituting equation 6 into equation 7 and comparing with equation 9 we find

$$K \propto f_0/f_1 - 1 = D \quad (10)$$

where we define $f_0 = f(n_1)$ and $f_1 = f(\theta)$. Therefore, the assumption in equation 6 states that the measurable cell polarity $f(n)$ changes logarithmically with n . As shown in Table 2, the additional experimental data of three cell lines at four different densities point to this statement. Finally, substituting equation 6 into equation 7 and the subsequent result into equation 8, we return approximately to the Gompertz equation in its differential form

$$dn/dt \approx -(f_0/f_1 - 1) \cdot n \cdot \ln(n/\theta) \quad (11)$$

which clearly contains the statement that describes $D \propto K$.

Table 2. Polarity as a function of the cell density in A549, NCI-H596 and NCI-H520 cell lines

A549		NCI-H596		NCI-H520	
Density ^a	Polarity ^b	Density ^a	Polarity ^b	Density ^a	Polarity ^b
20	3.72 ± 0.19	16	3.10 ± 0.34	13	1.82 ± 0.08
40	2.97 ± 0.35	34	2.57 ± 0.55	49	1.72 ± 0.14
95	2.37 ± 0.15	98	2.17 ± 0.12	155	1.57 ± 0.12
140	1.96 ± 0.17	160	2.02 ± 0.21	330	1.37 ± 0.09

^aCell density are given as cells/cm² × 10³. ^bPolarity values (f) were obtained by measuring the largest (r_i) and the smallest (r_j) diameter of > 80 cells of each line at four different densities ($f = r_i/r_j \pm$ SD).

DISCUSSION

The current study presents a Gompertzian model for cell growth as a function of cell phenotype. Using six human cell lines (A-549, NCI-H596, NCI-H520, HT-29, SW-620 and U-251) we have characterized a cell morphological parameter (named deformation coefficient D) that describes the increase in cell number with time, establishing a morphological interpretation of an empirical function widely used to fit biological growth (Turner *et al.* 1976; Bajzer & Vuk-Pavlovic 1996).

In order to estimate growth pattern by morphological measurements, we may consider that cell shape represents a visual manifestation of an underlying balance of forces and chemical signs (Olive & Durand 1994). Accordingly to Ingber (1999), cell spreading alone was conducive to proliferation and increases in DNA synthesis, indicating that cell morphology is a critical determinant of cell function, at least in the presence of optimal growth factors and extracellular matrix (ECM) binding. In many cells, the changes in morphology can stimulate cell proliferation through integrin-mediated signalling, such as MAPKs, which leads to growth only when cells adhere to and spread on the substrate, indicating that cell shape may govern how individual cells will respond to chemical signals (Boudreau & Jones 1999).

Acknowledging these observations, we have proposed the use of cell distortion and projection for modelling cell growth kinetic. The relationship among incidental diameters expressed by the cell deformation coefficient D gives the amplitude of cell distortion, which has been shown to be highly correlated with the Gompertzian growth parameter. Many authors have attempted to explain the Gompertz model in the context of cell growth, i.e. cell kinetics (Frezen & Murray 1986), cellular quiescence (Gyllenberg & Webb 1989), entropy (Calderón & Kwembe 1991), tumour heterogeneity (Kendal 1985) and cell interaction (Momback *et al.* 2002). According to Vinay & Alexandro (1982), one of the major drawbacks of the Gompertz model is that the original equation is not derived from a physiological basis. Besides the well-established statement of the Gompertz model (successfully fitted to many experimental data), its function lacks biological interpretation (Norton *et al.* 1976; Marusic *et al.* 1991; Marusic *et al.* 1994). Therefore, the mechanisms connecting morphology and population growth of tumour cells may provide an alternative view on the Gompertz model. Indeed, it also could be expected that any of a wide variety of retarded growth curves, i.e. logistic, Bertalanffy, Richards, etc., could provide an equally satisfactory correlation to a specific phenotype, although the establishment of new parameters may be necessary. This assumption, however, needs further investigation, including a comparative study of the phenotypic pattern described in this paper, with possibly interrelations that may exist among the biological-based growth models.

In conclusion, using a well-established growth model, our study supports the idea that cell growth kinetics can be modulated by phenotypic organization of attached cells and also attempts to understand the Gompertzian growth in the cellular structure level. The ability to estimate the growth pattern of an individual tumour cell type on the basis of morphological measurements should have general applicability in cellular investigations. For example, it could point out particular cell types to study cell-growth kinetics, cell transformation and morphogenesis.

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