Oscillations in growth of multicellular tumour spheroids: a revisited quantitative analysis

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Abstract

Objectives: Multicellular tumour spheroids (MTS) provide an important tool for study of the microscopic properties of solid tumours and their responses to therapy. Thus, observation of large-scale volume oscillations in MTS, reported several years ago by two independent groups (1,2), in our opinion represent a remarkable discovery, particularly if this could promote careful investigation of the possible occurrence of volume oscillations of tumours ‘*in vivo*’.

Materials and methods: Because of high background noise, quantitative analysis of properties of observed oscillations has not been possible in previous studies. Such an analysis can be now performed, thanks to a recently proposed approach, based on formalism of phenomenological universalities (PUN).

Results: Results have provided unambiguous confirmation of the existence of MTS volume oscillations, and quantitative evaluation of their properties, for two tumour cell lines. Proof is based not only on quality of fitting of the experimental datasets, but also on determination of well-defined values of frequency and amplitude of the oscillations for each line investigated, which would not be consistent with random fluctuation.

Conclusions: Biological mechanisms, which can be directly responsible for observed oscillations, are proposed, which relates also to recent work on related topics. Further investigations, both at experimental and at modelling levels, are also suggested. Finally, from a methodological point of view, results

obtained represent further confirmation of applicability and usefulness of the PUN approach.

Introduction

Multicellular tumour spheroids (MTS) are aggregates of tumour cells that can be cultured *in vitro* under controlled conditions. They represent an experimental model of intermediate complexity between standard two-dimensional monolayer cell cultures and tumours *in vivo*. They have been extensively used to investigate microscopic properties of solid tumours and their responses to different types of therapy (3–5). In addition, growth dynamics of MTS in many regards follow ones observed for real tumours. For this reason, MTS have also been exploited to test validity of growth models in an attempt to extract predictions *in vivo*, possibly in view of optimized control of tumour growth (6–13). MTS have an advantage over other experimental models in that their volume can be measured with great precision and for extended time periods.

During a detailed survey of MTS growth dynamics performed some years ago (1,14), unexpected growth patterns have emerged, consisting of low-frequency periodic oscillations of MTS size superposed to power-law correlated noise. Similar oscillations have also been observed by Deisboeck *et al.* (2) for MTS grown in specific extracellular matrix gel, which, contrary to a fluid host, prevented spontaneous MTS volume expansion by exerting mechanical constraint on volume of the proliferating cell mass. Oscillations were explained (in this case) in terms of nonlinear feedback between proliferation, occurring at the MTS surface, and invasion of the surrounding semi-solid environment, by tumour cells leaving the spheroid surface. Overall, these observations support the notion of MTS as dynamic self-organized biosystems (2,14), and reveal the not yet fully explored complexity of their growth at the microscopic level.

To the best of our knowledge, no other experiments have addressed the complex growth behaviour of MTS

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and results mentioned here are still the only available data for further investigation of mechanisms involved. Since some of these mechanisms could also be relevant to tumours *in vivo*, we believe that further analysis of the data, in particular from Chignola *et al.* (1,14) might indeed be very useful, since they have included extensive datasets represented by time series of spheroids' size, sampled daily over long time periods (60–70 days).

In the above-mentioned studies, no microscopic observations were reported to support or explain the results, as in the study of Deisboeck *et al.* (2), and techniques from standard signal processing theory (FFT) were applied to analysis of the residuals, on fitting growth data with the Gompertz model. They identified a major peak in power spectra for all series, hence presence of a main harmonic with an average period of 24.4 ± 15.5 and 15.4 ± 6.4 days for 9L and U118 MTS, respectively. This means that, on average, only 2.5–4.5 cycles were observed in each series and thus one may wonder about the statistical meaning of the results. Unfortunately, MTS cultures could not be extended any longer due to cell death and structural instability of spheroid structure.

Some evidence of volume oscillations has been found in a few previous studies on MTS, although this was not mentioned by other authors. For example, in a 1982 study (15), oscillations were observed in growth of heat-treated EMT6 Ro spheroids in syngeneic recipients, although they 'disappeared' when number of clonogenic cells per spheroid was plotted (see their Figs 3 and 4). Some evidence of possible oscillations was also included (but not mentioned) by Freyer and Schor (16).

We believe that a different and independent approach can be very useful to corroborate these previous results

and shed new light on biological mechanisms underlying MTS oscillations. The goal of the present contribution has been to take advantage of a newly proposed approach for interpretation of experimental datasets (based on formalism of phenomenological universalities: PUN), in order to build a mathematical model of observed oscillatory growth behaviour of MTS. In spite of large error bars in the data, the model allows performance of a quantitative analysis that unambiguously demonstrates presence of oscillations and determines their properties.

It is worth mentioning that Byrne (17), inspired by previous experiments with endometrial cells, explored effects of time delays in cell proliferation and cell loss, both resulting from time-dependent interchange of molecular signals. Intriguingly, she showed that periodic oscillations of MTS radius can arise from delayed apoptotic cell removal, providing the first clues concerning biological mechanisms regulating onset of oscillations in MTS growth. However, to the best of our knowledge, there are no experiments aimed at measuring half-life of apoptotic bodies in spheroids, of cells *in vitro* or even of cells in tumour tissues *in vivo* [the latter process being carried out by phagocytic cells with important physiopathological implications (18)]. Thus, we cannot compare conclusions by Byrne with ours on a rigorously quantitative basis.

PUN methodology is concisely illustrated in the next Section together with the experimental set-up and techniques used for growth of tumour cells in culture medium. Then, in the following Section, results for the two tumour cells lines 9L and U118 are reported and analysed. Finally, in the last Section, an interpretation of the results is presented, in view of a search for biological mechanisms

Figure 1. Representation of the 32 series of MTS growth curves belonging to the tumoral line 9L [data from (1,14)]. Although the separation is by no means unambiguous, they seem to fall into four families, according to the asymptotic values of their volume. The latter may be obtained by fitting the curves with Gompertz (or U1) functions. The result of this extrapolation is reported in (b), where we have created a histogram of the distribution of their values. It can be noted that four different peaks are recognizable with an approximately normal distribution around each of them.

capable to explain, and possibly predict, the observed oscillations.

Model and methods

PUN approach

The phenomenological universalities (PUN) approach has been developed (19,20) as a new epistemological tool for discovering, directly from experimental data, formal similarities in different fields ranging from physics to biology to social sciences. The main idea is that, in totally different contexts, growth (or evolution) of a ‘body’ can be described by the same function $y(t)$ of the variable y (which can be, for example, mass or length or height, or further parameters of the body) *versus* time t , because of some common features in the datasets. Growth rate a may vary over time in a complex nonlinear manner, possibly reflecting different phases exhibiting new and different metabolic stages of interplay with the host. For example, for tumours, initial avascular growth (solely by means of diffusion of nutrients) is followed by start of new mechanisms such as host invasion and angiogenesis.

PUN formalism is briefly described in Appendix A. We limit ourselves here to recall that, in such an approach, relationships among main variables of the system (for example, between growth rate a and its time derivative b) are assumed in the form of power expansions, such as

$$b = \sum_{n=1}^{\infty} \alpha_n a^n = \beta a + \gamma a^2 + \dots \quad (1)$$

If a satisfactory fit of experimental data is obtained by truncating the above set at the N th term (or power of a), then we state that underlying phenomenology belongs to the universality class UN. For example, it can be easily shown (19,20) that the universality class U0 (that is, with $N = 0$) represents autocatalytic growth; U1 ($N = 1$) yields the well-known ‘Gompertz’ law, which has been used for more than a century to study all kinds of growth phenomena and U2 is the generalized form of West-like growth law (19,20). These are summarized in Table 1, which reports explicit expressions of $b(a)$, $z(t) = \ln(y(t))$ and a biological interpretation of parameters involved for the three classes.

With reference to parameters defined in Table 1, we note that U0 depends only on parameter a_0 , which is proportional to doubling time, assumed to be constant in linear treatment. However, it is often tempting, as a simple minded but sometimes useful approximation, to consider growth rates as being constant. One of the most complete and recent studies of growth processes (21) shows that, at least for lymphoblasts, growth rates vary with both cell size and cell cycle, and are therefore far from being constant. Time dependence of doubling comes naturally, in the framework of PUN formalism, as a result of transition from U0 to U1 and/or U2.

In addition to a_0 , in the class U1, there is an additional parameter β , which represents the retardation factor (changed in sign) that bends exponentially growing U0 curve towards saturation value $y_{\infty} = \ln(z_{\infty})$. Such a retardation factor is related to growth limitations imposed by the host, for example, the so-called ‘carrying capacity’. It is important to recall that class U1 is equivalent to the Gompertz law (22), whose applications have been shown to be ubiquitous in biology. For tumours, it has been assumed to be the basis of an important therapeutic approach known as the Norton–Simon hypothesis (23).

Finally, class U2, which represents a generalization of both the so-called West law (24) and the logistic law (25,26), includes one more parameter γ , which is related to growth exponent p by the simple relationship $\gamma = p - 1$. Parameter p , reflecting fractal dimension of the vascular network, has been shown to take different values depending on the stage of development of tumours (27). A ‘dynamic evolution model’ of power exponent p has been proposed by Guiot and collaborators, to take into account all stages of tumour evolution from avascular to angiogenetic. Ranges of values of parameters a_0 , β and γ (or p) have been reported for various applications (27,28). Time dependence of involved parameters has also been recently considered (29).

MTS generation

Detailed experimental procedures for MTS generation have been described previously (1). Briefly, approximately 10^6 cells from exponentially growing cultures of 9L (rat glioblastoma) or U118 (human glioblastoma) cell

Table 1. Explicit expressions of the generating functions $b(a) = \dot{a}$, growth rate $a(z(t))$ and $z(t) = \ln[y(t)]$ for the three classes U0, U1 and U2

Class	$b(a)$	$z(t)$	Parameter interpretation	Reference
U0	0	$z = a_0 t$	$a_0 = a(t = 0)$ specific growth rate, i.e. it represents doubling time divided by $\log(2)$.	(51)
U1	βa	$z = (a_0 / \beta)(\exp(\beta t) - 1)$	β retardation factor changed in sign and is defined as: $\beta = -a_0 / z_{\infty}$, where z_{∞} is the asymptotic value of $z(t)$, related to the host carrying capacity.	(6)
U2	$\beta a + \gamma a^2$	$z = -\frac{1}{\gamma} \ln \left[1 + \frac{a_0 \gamma}{\beta} (1 - e^{\beta t}) \right]$	$\gamma = p - 1$, where p is the growth exponent [see eqn (A4)]	(38)

Figure 2. (a) Fitting by means of eqn (A13) of representative instances of the four families of MTS growth curves belonging to the tumour cell line 9L [data from (1,14)]. The oscillations can be observed for all four families, in spite of the large experimental error bars. (b) detailed representation of one of the four curves (the second one from the top) and results of a U1 (Gompertz) and U1/T1 fitting. The huge improvement in the R^2 demonstrates that the oscillations really exist. Similar results have been obtained for all the other 9L MTS growth curves. (c) Additional proof of the existence of the oscillations comes from the FFT spectrum of the curve.

Figure 3. Histograms of the frequency ω , amplitude A and U1 parameter β : a, b and c, respectively. It is remarkable that all histograms can be fitted nicely by Gaussian functions, whose mean values and standard deviations are reported at the top of each plot.

lines were resuspended in 10 ml of RPMI medium supplemented with 10% heat-inactivated foetal bovine serum (FBS), and layered on agar (10 ml of a 0.75 w/v solution of agar in RPMI-FBS) in Petri dishes. Spheroids of roughly 200–250 µm diameter were harvested by gentle repeated transfer of individual spheroids (using a micropipette) into wells of 24-well culture plates. MTS were then individually placed into the wells of culture plates containing 1 ml RPMI-FBS 10% on a layer of 1 ml of 0.75 (w/v) agar in the same medium. Once in 7 days, 0.5 ml of growth medium was gently removed from each well and the same amount of fresh complete medium was added.

Spheroids were measured using a calibrated ocular micrometer with an inverted microscope. At best resolution, one unit of metric scale corresponded to 34 µm. Longest spheroid diameter (D) and perpendicular diameter (d) were measured; volume (V) was then calculated according to the formula $V = 4/3\pi r^3$, where r was mean radius of the spheroids.

Results

Results for the tumour cell line 9L

Results of measurements of volume *versus* time for 32 MTS growth experiments performed with cells obtained from tumour cell line 9L are reported in Fig. 1 [data from (1,14)]. By fitting each curve with a Gompertz (or U1) function (see the Appendix A), it appears that, albeit not unambiguously, the 32 curves may be separated into four families, according to asymptotic value of volume. Figure 1b, has four different peaks recognizable with near-Gaussian distribution around each. This ‘grouping’ of the 32 series is remarkable, as experiments were performed (see Models and methods) as much as possible always using the same procedure, so that separation into ‘families’ should be due, if confirmed, to ‘cloning’ of tumour cells themselves, or to presence of cell subpopulations with different proliferative potentials. Indeed, previous analysis of growth kinetics of 9L MTS, using a non-parametric method to define degree of similarity between growth curves, showed that they could be grouped into four to five subsets with similar growth kinetics within a large range of Euclidean distance between data points (30).

By applying eqn (A13), the U1/T1 fitting function, to the dataset, it is possible to obtain, by means of standard minimization procedure, parameters that describe the oscillatory part of the function. As an example, Fig. 2a shows four different cases belonging to one each of the four families hypothesized in Fig. 1. One of the four (third from top) is shown in more detail in Fig. 2b. By adding oscillatory parts to fitting function, one can obtain great

improvement in the value of R^2 (from 0.76 to 0.90). Another proof of existence of oscillations comes from a fast Fourier transform (FFT) of the growing curve, which exhibits, in spite of few and scattered data, a very peaked spectrum, with central frequency very near to that obtained with the fitting function (Fig. 2c). Systematic fits of all other curves belonging to the line 9L yield similar results.

Having proved that MTS growth oscillations really exist, we now turned our attention to investigation of their properties, that is their frequency ω , amplitude A and U1 parameter β (see Table 1). All these quantities were centred around well-defined ‘peak’ values with good Gaussian distribution around them (see Fig. 3a–c). This is quite remarkable due to the small number of cases and that the ‘occurrences’ must obviously be integers. Additional analysis, performed separately for the four ‘families’, which have been hypothesized in Fig. 1, shows that the Gaussian parameters do not depend appreciably on the group to which they belong. Indeed, this can be considered as further proof of existence of the oscillations, as, if they happened to be merely statistical fluctuations, corresponding values of parameters would be randomized.

Because of basic significance of a well-defined frequency ω for MTS growth oscillations, we have analysed in more detail the spectra of the growth curves to search for a secondary frequency ω' . The result was (see Fig. 4a) that, in the spectra, the second peak in amplitude falls at about $\omega' = 2\omega$ in almost all the cases, that is, other frequencies present in spectra are higher harmonics of the fundamental one. This has suggested to us to fit the curves by means of the U1/T2 fitting formula [obtained as a generalization of eqn (A13) in Appendix A]:

$$y = \left\{ y(0) \exp \left[\frac{a_0}{\beta} (1 - \exp[\beta t]) \right] \right\} \{ \exp[A_1 \sin[\omega t + \phi_1]] \} \{ \exp[A_2 \sin[2\omega t + \phi_2]] \} \quad (2)$$

with a further considerable improvement in the value of R^2 (see Fig. 4b).

To conclude, we remark that the value obtained for the peak frequency value $\bar{\omega} = (0.296 \pm 0.090) \text{ days}^{-1}$ corresponds to a period $\bar{T} = (22 \pm 8) \text{ days}$, in agreement with results of qualitative analysis of Chignola *et al.* (1).

Results for tumour cell line U118

For the line U118, there did not seem to be any indication of existence of distinct ‘families’ characterized by different asymptotic values of volume. MTS growth was followed for only 35 days, so that no saturation in volume was observed. However, by fitting the data with Gompertzian curve U1, it is possible, as in the previous case, to

Figure 4. By studying the spectra of the growth curves, it is possible to extract information about the presence of additional oscillation frequencies. Indeed, by plotting the value ω' of the second peak in amplitude in the FFT of the growth curve versus the fundamental frequency ω , we see that almost all the data fall on a straight line of slope 2 [see (a)], i.e. $\omega' = 2\omega$. In fact, by adding in the fitting a second harmonic [see (b)], a considerable improvement in the R^2 value may be achieved (from 0.90 to 0.96).

calculate asymptotic value of the volume. Results, even if the number of datasets is not large, seem to confirm existence of a unique group with central value $V_{\text{asympt}} = (212 \pm 64) \times 10^{-3} \text{mm}^3$.

We then proceeded as before to the analysis of oscillations in volume of the MTS (see Fig. 5). By performing a U1/T1 fitting of the datasets, we found that oscillation frequency ω was, also in this case, well defined with a value of $\bar{\omega} = (0.39 \pm 0.11) \text{days}^{-1}$, corresponding to $\bar{T} = (16 \pm 5) \text{days}$, and $\beta = (-0.11 \pm 0.06) \text{days}^{-1}$. It is interesting to perform Student's t-test to verify whether mean values of the two normally distributed populations (9L and U118) coincided. We obtained a t value of 3.24 for 46 degrees of freedom. With a confidence level of 99%, we could then state that the two mean values are different, and the data belong to two different populations. Hence, the value of the parameters seemed to depend on the tumour line, and not on properties of the surroundings (for example, the culture medium) or on experimental artefacts.

Discussion

Using a recent approach, based on formalism of phenomenological universalities (19,20), we have confirmed the existence of a well-defined oscillatory pattern in the MTS growth curves (1,14). The MTS oscillations, which are superimposed on behaviour well described by the universality class U1, are not trivial fluctuations, but real features capable of yielding new information about tumours. Not

only existence of the oscillations, but also the significance of their parameters (and of those of U1 and U2), represent novel tools for gaining a better understanding of tumoral growth. Physical and/or biological origins of such oscillations remain, however, to be explained.

To be sure, periodicities and oscillations are ubiquitous in biology (and in all other sciences); they can be easily explained based on a feedback between an individual 'body' and its environment (or between competing populations). We wish, however, in the following, to go beyond a merely mathematical model and explore plausible biological mechanisms, which can be directly responsible for observed oscillations. Let us note that, based on our PUN approach, such oscillatory behaviour could mainly affect growth rate (for U0, U1 and U2), the host's carrying capacity (for U1 and U2) and the fractal dimension of the distributive network (for U2). Thus, oscillatory behaviour might result from different biological mechanisms.

We first observe that the host's carrying capacity can be limited by various mechanisms related to both tumour/environment, mechanical and metabolic interactions. We have therefore investigated whether a mechanical-type interaction between MTS and their environment may be responsible for the observed oscillations, as proposed by Deisboeck *et al.* (2). In their case, however, MTS were cultured in gel instead of in a liquid medium, and their growth – inhibited by the semisolid environment – was observed for a much shorter time span, during which oscillations for a period of about 4–5 days were observed. Liquid environment, such as the one utilized in

Figure 5. Two instances of comparison between a U1 and U1/T1 fit for MTS growth curves belonging to the tumour cell line U118 [data from (1,14)]. As in the case of the line 9L, a large improvement may be obtained by allowing for the presence of well-defined oscillations.

the experiments considered here, cannot exert any elastic effect on the growing MTS. It is, however, conceivable that, under the effect of the increasing internal pressures exerted by mitosis, cohesion of superficial cells (a sort of surface tension, partly due to cell–cell adhesion on the surface and partly to the genuine tension of the liquid membrane formed at the MTS–culture medium interface) might be critically challenged.

According to Laplace laws, which have already been evoked in explaining tumour structure elsewhere (31), surface cohesion might be restored provided a proper reduction of the internal pressure occurs. This might result from cell shedding, but unfortunately, although cell shedding has been observed during the growth of MTS, there was no way to perform systematic direct measurements in the experiment being considered here. Cell shedding from MTS was, nonetheless, observed and measured in the study of Landry *et al.* (15), where it was also shown that the process could be markedly accelerated by hyperthermia. Spheroid volume oscillations were consequently affected.

Interactions of MTS with its environment may also be related to its metabolic conditions, e.g. to nutrient availability (32–34). In an interesting study in another context, that is, spatio-temporal patterns of formation of bacterial colonies, Lacasta *et al.* (35) observed many different growth patterns, and reproduced them by means of a diffusion–reaction model. They found that, for a narrow region of diffusion parameters, colonies developed a ‘ring pattern’, exhibiting regular oscillations in cluster radius and in radial density profile. Intriguingly, the same diffusion–reaction equations were the starting point for analysis of MTS growth in the investigation by Garner *et al.* (36). They assumed allometric law proposed by West

et al. (37,38), [and extended to tumours by Guiot *et al.* (39) and corresponding to the PUN class U2 (40)], to describe volume growth of MTS by incorporating in the model the local nutrient concentration. By coupling the allometric law with the assumption that the nutrient is distributed by diffusion, they predicted the formation of an inner necrotic core, in agreement with the experimental observations. A steady state is predicted, but, if we allowed for a longer time, the necrotic cells could decompose, possibly supplying nutrients to the inner layers of the MTS and temporarily restoring their availability, so that volume oscillations could occur. Once again, however, there are no quantitative data on microscopic interactions of cells in the inner layers of the MTS. Information in this sense might result from multi-scale models of MTS growth, which are presently under development [such as in Ref. (41)].

Finally, let us point out to another possible mechanism that, as far as we know, has received little or no attention: in parallel to the hypoxic conditions in the MTS core, hyperoxic conditions are normally experienced by the cells of the outer layers of the MTS, and this is due to the fact that cells are cultured at ambient oxygen concentration (e.g. 20% oxygen concentration in the medium), which is much higher than in human tissues (e.g. 1–6%). This might result in oxidative stress, causing cell senescence in the outer layers. In fact, oxygen concentrations in the physiological range of 1–6% have been measured in spheroids approximately 100 μm below the surface, i.e. well inside the proliferative layer (42), and cell death has been observed to occur even in the rim of viable cells (4). Although tumour cells have been generally considered immortal, recent studies have questioned this assumption (43). Cellular senescence, which entails a limitation in the

number of times a cell can replicate itself, has been observed under different conditions. In particular, it can be induced by telomere shortening following oxidative damage, a process that can reduce up to 15–20 the number of duplicating cells (44). Such a mechanism might explain why, starting from a positive growth rate, the outer cells could die and reduce MTS volume. This mechanism, coupled with the experimentally observed (45,46) cell migration to the inner layers of the MTS and with the decomposition of dead cells, would generate a feedback loop responsible for a cyclic process, with a periodicity T related to the average number of cycles before cell senescence and chemical decomposition. Assuming a cell cycle lasting 24 h, an oscillation period of 22 days – such as that obtained in the present study – would correspond to a cell life of around 20 cycles, which is comparable with data from cell senescence studies (44,47). The occurrence of a ‘macroscopic’ oscillating pattern would obviously require a synchronization of cell cycles. Although cells tend to normally desynchronize with the progression of mitotic division (48), cell synchronization can, however, be achieved by different mechanisms (49) including some modifications of the culture environment. Moreover, the progressive desynchronization modelled by Milotti *et al.* (48) is much more evident for dispersed cells than for cell clusters, where spatial correlations develop to improve the structural stability of the spheroid.

To step from speculation to scientific evidence requires more experimental work to be performed, and multi-scale models reproducing the microscopic interactions between tumour cells and host must be elaborated. The aim of the present study was to stimulate such efforts, showing that also the various microscopic scenarios proposed above can be incorporated in the framework of Phenomenological Universalities.

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Appendix A

In the present context, Phenomenological Universalities are formulated as a method for solving the following problem: given a string of data $y_i(t_i)$ and assuming that they refer to a phenomenology, which can be reduced to a first order ODE of the type

$$\dot{y}(t) = a(y, t)y(t), \quad (A1)$$

where a dot denotes a time derivative, we search for a solution $y(t)$, based not on simple numerical fitting, but on

an unbiased (i.e. absolutely interdisciplinary or universal) procedure.

It may be useful to introduce the auxiliary variable $z = \ln(y)$, so that eqn (A1) becomes

$$\dot{z} = a(z, t) \quad (A2)$$

Starting with the case $a = a(z(t))$, i.e. assuming a nonlinear but not directly time-dependent rate of growth, the problem of finding $z(t)$ and $y(t)$ is reduced to the integration first of the equation $\dot{a} = b$, with b given by eqn (1), truncated at a given N , and then of eqn (A2). The results are summarized in Table 1, for $N = 0, 1$ and 2 (U0, U1 and U2, respectively).

For the class U2, it can be easily proved that the solution

$$y = e^z = [1 + \psi(1 - e^{\beta t})]^{-1/\gamma} \quad (A3)$$

where $\psi = (\gamma/\beta)a_0$, satisfies the ODE

$$c_1 y^p = \frac{dy}{dt} + c_2 y \quad (A4)$$

where $p = 1 + \gamma$, $c_2 = \beta/\gamma$, $c_1 = c_2(1 + \psi)$.

In many applications, a very important interpretation can be attached to eqn (A4). That is, we may consider $c_1 y^p$ as the energy input, dy/dt the growth of the system and $c_2 y$ the energy consumption (e.g. due to the metabolism). Saturation (i.e. no further growth : $dy/dt = 0$) follows when $c_1 y^p = c_2 y$. This interpretation, which is also important in the context of therapeutical dosages (50), implies that eqn (A4) is an expression of the energy conservation of the system (or of the First Principle of Thermodynamics). It also suggests that, as a direct result of the nonlinearity of eqn (1), fractality may follow. In the model of West and collaborators, $p = 3/4$. In a recent model by C. Guiot *et al.* (27), the exponent p varies between a first avascular phase of tumoral growth and the onset of angiogenesis.

Next, let us consider the case

$$a(z, t) = \bar{a}(z) + \tilde{a}(t), \quad (A5)$$

where a is assumed to be the sum of two contributions to the growth rate, one ($\bar{a}$), that depends only on z (or y) as in UN, while the other ($\tilde{a}$) is solely time-dependent.

In eqn (A5) the term $\tilde{a}(t)$ can be interpreted as the interaction of $y(t)$ with its host (or environment) $Y(t)$. In fact, such an interaction yields in the RHS of eqn (A1) a term proportional to Y :

$$\dot{y} = a(y)y(t) + A(Y)Y(t) \quad (A6)$$

Assuming, as in a ‘Gedankenexperiment’, to solve for both $y(t)$ and $Y(t)$ (e.g. by solving a system of coupled equations) it is possible, at least conceptually, to define

$$\tilde{a}(t) = A(Y(t))Y(t)/y(t) \quad (A7)$$

i.e., $\tilde{a}$ is related to the host carrying capacity, which limits the tumour asymptotic mass.

Then, by writing

$$\dot{a} = \tilde{a}(\bar{y})\bar{y}, \quad (A8)$$

it follows

$$\dot{y} = (\bar{a} + \tilde{a})\bar{y}\bar{y}, \quad (A9)$$

Equation(A1) can be split into a system of two uncoupled equations

$$\dot{\bar{y}} = \bar{a}(z)\bar{y} \quad (A10)$$

and

$$\dot{\tilde{y}} = \tilde{a}(t)\tilde{y} \quad (A11)$$

Equation(A10) can be solved as before giving rise to the UN classes. A general solution of eqn (A11) can be written as

$$\tilde{a} = \dot{\tilde{z}} = \sum_{n=1}^{\infty} A_n E_n \quad (A12)$$

where $E_n = \exp[i(n\omega t + \phi_n)]$. If the sum in eqn (A12) can be truncated at the M th term, we will state that the corresponding phenomenology belongs to the class UN/TM. In the simplest case, one can easily find that the U1/T1 solution is given by

$$y = \left\{ y(0) \exp \left[\frac{a_0}{\beta} (1 - \exp[\beta t]) \right] \right\} \left\{ \exp[A_1 \sin[\omega t + \varphi_1]] \right\}. \quad (A13)$$

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