Collective behavior of brain tumor cells: The role of hypoxia

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We consider emergent collective behavior of a multicellular biological system. Specifically, we investigate the role of hypoxia (lack of oxygen) in migration of brain tumor cells. We performed two series of cell migration experiments. In the first set of experiments, cell migration away from a tumor spheroid was investigated. The second set of experiments was performed in a typical wound-healing geometry: Cells were placed on a substrate, a scratch was made, and cell migration into the gap was investigated. Experiments show a surprising result: Cells under normal and hypoxic conditions have migrated the same distance in the “spheroid” experiment, while in the “scratch” experiment cells under normal conditions migrated much faster than under hypoxic conditions. To explain this paradox, we formulate a discrete stochastic model for cell dynamics. The theoretical model explains our experimental observations and suggests that hypoxia decreases both the motility of cells and the strength of cell-cell adhesion. The theoretical predictions were further verified in independent experiments.

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I. INTRODUCTION

Collective behavior of living cells [1] is an intriguing and challenging phenomenon not only from the biological perspective, but also from the physical point of view. This explains an increasingly growing interest about this subject in the physics community [2]. A remarkable range of collective behavior is displayed in morphogenesis [3], wound healing [4], and tumor growth [1,4,5]. In the present study, we focus on the invasion of aggressive malignant brain tumors [6].

Gliomas are the most common type of primary brain tumors [7]. A hallmark of malignant gliomas is their ability to invade into surrounding brain tissue. Glioma cells not only proliferate (divide), but detach from the tumor core and actively migrate away into the extracellular matrix [4]. Therefore, surgery is commonly noncurative as cells invade the brain and escape resection [8]. The invasive nature of malignant brain tumors often leads to recurrence: formation of distant recurrent (secondary) tumors in the invasive region. Experimental observations show that cells on the surface of a primary tumor (so-called proliferative cells) divide much more frequently than individual invasive cells [8,9]. However, invasive cells might form clusters, which can eventually develop into secondary tumors.

In experiments, the growth of glioma spheroids in transparent gel has been investigated. The observed patterns are different for two cell lines; the mutant cells (U87-ΔEGFR) form small clusters within the invasive region, while the wild type cells (U87) do not cluster [10,11]. More strikingly, when cells are placed on a substrate, wild type cells remain homogeneously distributed over the system, while mutant type cells eventually form clusters, which grow with time [12]. Using discrete stochastic modeling [12,13], we have shown that radically different morphologies can result even from small differences in the strength of cell-cell adhesion. When the adhesion parameter becomes larger than its threshold value, the uniform system becomes unstable, which leads to phase separation: Clusters coexist with individual migrating cells. This idea was inspired by a very different physical phenomenon: a ferromagnetic phase transition in the Ising model [14]. Importantly, the theoretical predictions were verified in a separate experiment [12].

The main focus of the present work is on an intriguing question: How does hypoxia (lack of oxygen) affect cell behavior? Cells inside a primary brain tumor are regularly subjected to hypoxic conditions. It is well known that hypoxia leads to increased tumor invasion [15]; however, the mechanism of this phenomenon remains unclear. How do hypoxic conditions affect cell motility, cell proliferation, and cell-cell adhesion? We investigated these problems both experimentally and theoretically; we further confirmed our theoretical predictions in additional experiments.

II. EXPERIMENTS

A series of experiments were performed with cells cultured under normoxic (20 percent oxygen) and hypoxic (less than 1 percent oxygen) conditions. First, we measured the proliferation rate of normoxic and hypoxic cells. There, cells were placed uniformly on a substrate, and the cell density was measured as a function of time. In all the experiments we performed, culture wells were polystyrene, modified by incorporation of oxygen ions, producing a surface that becomes hydrophilic and negatively charged when medium was added, which enabled cell attachment and spreading. We checked that the cells remain uniformly distributed over the system and did not form clusters [12]. We fitted the experimental growth curves with the modified logistic growth formula, solving the equation

$$\frac{du}{dt} = \alpha(u)u,$$  \hspace{1cm} (1)

where the rate of proliferation depends on the local area fraction (normalized cell density) $0 < u < 1$. There are many ways to model the density dependence of the proliferation rate. For example, one can consider $\alpha(u) = \alpha (1 + u)^\beta (1 - u)$. Here, $\beta = 0$ gives the usual logistic growth formula; it is known, however, that the proliferation rate is more or less constant at small and intermediate densities and decreases at high densities due to contact inhibition effect. Another
FIG. 1. (Color online) U87 glioma cell proliferation on a substrate: cell number as a function of time. Symbols show experimental results for cell proliferation under normoxic (circles) and hypoxic (squares) conditions. A theoretical fit given by Eq. (1) for the two expressions for $\alpha(u)$: $\alpha_1(u)$, $\beta = 1.73$, solid lines and $\alpha_2(u)$, $\nu = 6$, dashed lines. The parameters are $u_0 = 0.14$, $\alpha = 1/29$ hour$^{-1}$ (upper solid line) and $\alpha = 1/53$ hour$^{-1}$ (lower solid line); $u_0 = 0.14$, $\alpha = 1/29$ hour$^{-1}$ (upper dashed line) and $\alpha = 1/50$ hour$^{-1}$ (lower dashed line).

The first naive attempt to attack this problem would be the following. Cell invasion is often modeled as propagation of fronts of cells that can be described by the Fisher-Kolmogorov
The lower panel shows a snapshot of the system 24 hours after closing the scratch. Initially cells were uniformly placed on a plastic substrate, and a 2-mm-wide scratch was made (upper panel). The distance that cells had migrated in 24 hours for both normoxic and hypoxic cells. Averaging over many experimental runs and over the lateral coordinate shows that normoxic cells have migrated the same distance (so that the velocity of migration is the same for both types of cells, Fig. 2). But then cells should have migrated the same distance in the scratch experiment as well. To explain this controversy, we should take into account the phenomenon of cell-cell adhesion.

**III. DISCRETE STOCHASTIC MODELING**

Agent-based modeling of cell migration has become increasingly popular in recent years [5,18]. Here we employ a discrete stochastic model for cell migration, introduced in [12,13]: the modeling incorporates not only cell proliferation and cell motility, but also cell-cell adhesion. The basic unit of the model is a single cell. Consider a two-dimensional square lattice (the same model can be formulated in three dimensions); each lattice site can be either empty or occupied by a single cell. The lattice distance is taken to be 20 μm, which is the effective diameter of a glioma cell. A randomly chosen cell can either proliferate to an empty neighboring site or migrate there, where the probabilities for proliferation and migration are determined from the experimentally measured rates of proliferation and migration. The probability for proliferation is given by the rate of proliferation times the characteristic diffusion time \(\bar{\alpha}(n) = \alpha(n) \) \(t_{\text{diff}}\), where the expression for \(\alpha(n)\) is discussed after Eq. (1). \(t_{\text{diff}}\) is the average time required for a cell to move the distance equal its own diameter (this time is of the order of 10 minutes), and \(n\) is the number of the nearest neighbors (which can vary between 0 and 4). The probability for migration is \([1 - \alpha(n)](1 - q)^n\), where \(q\) is the cell-cell adhesion parameter. The case \(q = 0\) represents no adhesion. The probability for migration decreases as the adhesion parameter \(q\) increases, since a cell needs to detach from its neighbors. The adhesion parameter \(q\) can be experimentally measured since it is related to the shed rate of invasive cells from the tumor surface [12]. Cells with \(q = 1\) will not detach from the tumor at all, so any measured shed rate corresponds to \(q < 1\). After each “single cell step,” the time is advanced by \(t_{\text{diff}}/N\), where \(N\) is the current number of cells. The model does not directly incorporate the cell-substrate (or cell-matrix) adhesion, which is an important factor in cell migration [19]; however, it is taken into account indirectly. Cell-substrate adhesion is known to affect cell migration [20]: therefore, a specific migration time \(t_{\text{diff}}\) corresponds to a specific strength of cell-substrate adhesion [21].

This model is now applied to describe the two main experiments we performed: (1) Cells migrate away from the spheroid and (2) cells migrate in the scratch wound geometry. The four unknown parameters of the model are the respective diffusion times and adhesion parameters for glioma cells under normoxic and hypoxic conditions: \(t_{\text{diff}}\), \(q_{\text{norm}}\), \(q_{\text{hyp}}\), and \(t_{\text{diff}}\), \(q_{\text{hyp}}\). The distance \(r\) that the cells have migrated depends on these parameters: \(r = r(t_{\text{diff}}-q)\). We performed extensive numerical simulations of the model, computing \(r\) for different sets of parameters in the two settings, keeping the proliferation rate \(\bar{\alpha}\) constant (assuming the experimentally found values for proliferation). In the scratch wound geometry, we averaged the results over many simulations and over the lateral coordinate. In the spheroid geometry, we averaged the results over many simulations and over the azimuthal direction.

Let us now plot curves \(r = \text{const}\) in the phase plane \((t_{\text{diff}}-q)\), where the value of the constants is taken from experiments.
Consider, for example, the scratch experiment with cells under normoxic conditions. In the experiment, cells migrated $r = 1020 \, \mu m$ in 24 hours. In the model, we choose a value of cell-cell adhesion parameter $q$ and compute $r$ for various diffusion times $t_{\text{diff}}$. Then we find the value of $t_{\text{diff}}$ for which $r = 1020 \, \mu m$. This gives a pair $(t_{\text{diff}}, q)$. Choosing a different value of $q$ we get a different value of $t_{\text{diff}}$. This procedure gives the curve $r = \text{const}$ in the $(t_{\text{diff}}, q)$ phase plane. Figure 4 shows four such curves. The upper panel describes simulations for cells under normoxic conditions: The dotted line corresponds to the spheroid experiment (Fig. 2), $r = 1300 \, \mu m$; the solid line corresponds to the scratch experiment (Fig. 3), $r = 1020 \, \mu m$. The lower panel describes simulations for cells under hypoxic conditions: The dotted line corresponds to the spheroid experiment (Fig. 2), $r = 1300 \, \mu m$; the solid line corresponds to the scratch experiment (Fig. 3), $r = 730 \, \mu m$. Notice that the role of adhesion in the scratch wound geometry is not high: The distance $r$ depends on $q$ rather weakly. In the case of spheroid experiment, the role of adhesion is higher, since cells first need to detach from the spheroid.

We seek parameters of the model that give the experimentally observed values of distance that cells have migrated. The region of probable intersection between the two curves on the upper panel of Fig. 4 determines parameters $(t_{\text{diff}}, q)$ for normoxic cells; the respective region on the lower panel of Fig. 4 determines parameters $(t_{\text{diff}}, q)$ for hypoxic cells. Comparing the two regions in the parameter space, one can see that $t_{\text{diff}}^{\text{norm}} < t_{\text{diff}}^{\text{hyp}}$ and $q^{\text{norm}} > q^{\text{hyp}}$. The model suggests therefore that hypoxia not only suppresses cell motility (in contrast to our naive expectations), but it also substantially reduces the strength of cell-cell adhesion. We find this result to be quite important: The decreased cell-cell adhesion allows hypoxic cells to detach from the tumor much easier, which leads to an enhanced invasion, despite the decreased cell motility.

Theoretical modeling is successful when it not only describes the existing experimental observations, but also can produce an experimentally testable hypothesis. One of the main theoretical predictions of our model is that the strength of cell-cell adhesion decreases due to hypoxia. To test this hypothesis, we performed a Western blot to measure expression of E-cadherin, an important transmembrane protein that positively regulates the strength of cell-cell adhesion, for example, in carcinomas [22] and gliomas [23]. E-cadherin is known to act as an invasion suppressor molecule [22,23]. Our western blot experiments showed a clear down-regulation of E-cadherin 24 hours after cells had been placed under hypoxic conditions. This finding clearly confirms our theoretical prediction, and is consistent with a recent evidence that hypoxia down-regulates E-cadherin in breast cancer cells [24], see also [25]. Another important cell-cell adhesion molecule is N-cadherin, the expression of N-cadherin is known to correlate with the ability of cells to form aggregates [19]. We performed another experiment to study the effect of hypoxic conditions on the expression level of N-cadherins. Western blot showed a clear downregulation of N-cadherins, similarly to what was observed for E-cadherins. Thus, we verified that hypoxia down-regulates cadherin expression decreasing the strength of cell-cell adhesion.

IV. SUMMARY AND DISCUSSION

In summary, we demonstrated a successful application of a physically inspired approach to complex biological multicellular dynamics. Using discrete stochastic modeling, we explored the correspondence between the “microscopic” characteristics of a cell (cell diffusion coefficient, cell proliferation rate, and the strength of cell-cell adhesion) and the emerging macroscopic collective behavior of these cells. Specifically, we reproduced the experimental findings on migration of glioma cells under normoxic and hypoxic conditions in two different geometries and predicted a decrease both in cell...
motility and in the strength of cell-cell adhesion due to hypoxia. We further confirmed the theoretical prediction on decrease of cell-cell adhesion in independent experiments. As we mentioned above, one can directly estimate the adhesion parameter \( q \) by measuring the shed rate of invasive cells from the tumor surface; this was done in Ref. [12]. It is known that cell-cell adhesion acts as a tissue surface tension [26], it would be interesting to find a relation between \( q \) and the surface tension of a tumor spheroid.

In this paper we investigated the effect of hypoxia employing discrete stochastic modeling. A promising approach in modeling biological systems is connecting the “microscopic” agent-based models and “macroscopic” continuum approach [5,27]. Let us consider the discrete model employed in the paper in case of zero adhesion, \( q = 0 \). Let us assume that the probability for proliferation is much smaller than the probability for migration. This is a reasonable biological assumption for glioma cells, since the typical time for migration is much smaller than the typical time for proliferation. In this case, the probability that the site \( n \) is occupied, \( P(n) \), and the probability that the neighboring site \( n + 1 \) (or the site \( n - 1 \)) is occupied, \( P(n + 1) \), are independent. Therefore, the master equation for cell dynamics (in one dimension) can be written in the form

\[
P(n,m+1) - P(n,m) = \frac{D}{2} \{ P(n-1,m)[1-P(n,m)] \} + \frac{D}{2} \{ P(n+1,m)[1-P(n,m)] \} - \frac{D}{2} \{ P(n,m)[1-P(n-1,m)] \} - \frac{D}{2} \{ P(n,m)[1-P(n+1,m)] \} + \bar{\alpha} \{ P(n-1,m)[1-P(n,m)] \} + \bar{\alpha} \{ P(n+1,m)[1-P(n,m)] \} \nonumber \]

\[
= \frac{1}{2} \{ [D + \bar{\alpha}][1-P(n,m)] \} \times \{ P(n-1,m) - 2P(n,m) + P(n+1,m) + \bar{\alpha} P(n,m) \} \]

In this paper, we studied cell migration on a substrate. Although this in vitro setting allows investigation of the behavior of glioma cells (for example, various aspects of cell motility [30], cell spreading [31], or cell aggregation [12]), the real in vivo geometry is three dimensional. To mimic in vivo tumor growth, the dynamics of multicellular tumor spheroids in gel can be considered [11,19,32]. Investigation of cell invasion in these systems should take into account matrix degradation [33]. This is an interesting direction of future research.

The concept of emergence and emergent behavior in biological systems has become increasingly popular in recent years [1,34]. This concept addresses self-organization of complex biological systems and emphasizes the qualitative difference between the collective (macroscopic) behavior of a large number of microscopic agents and the behavior of individual agents. It is often claimed that complex biological systems are irreducible, and emergent macroscopic behavior cannot be predicted or deduced from the microscopic behavior [35]. Here we show that this is not always the case. In our model, the “microscopic” objects are individual glioma cells, 10–20 \( \mu \)m in diameter (this is actually a mesoscopic scale with respect to scales inside a cell). Using the model, we successfully predicted the collective cell behavior on a “macroscopic” scale, of the order of millimeters, formulating a minimalist model with relatively small number of parameters that nevertheless captures the essential physics and biology. The model provides a connection between the “microscopic” properties of individual cells (like the strength of cell-cell adhesion) and the “macroscopic” properties of the emergent system (like the velocity of propagation of cell density fronts). Importantly, the model provided theoretical predictions that were further tested in experiments. We demonstrated that employing minimalist models (where their formulation is possible) can substantially simplify the analysis and understanding of the emergent collective behavior of complex multicellular systems.