Role of repulsive factors in vascularization dynamics

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(Received 21 February 2005; revised manuscript received 20 October 2005; published 14 April 2006)

Capillary networks are essential in vertebrates to supply tissues with nutrients. Experiments of in vitro capillary formation show that endothelial cells randomly spread on a gel matrix autonomously organize to form vascular networks with a characteristic length independent of the initial cell density. A mathematical model based on free cell migration and on cell cross-talk mediated by soluble chemical factors has been recently proposed and explains the main dynamical and geometrical properties of the networks. We extend this model introducing the action of repulsive factors and we show that their activity results in a larger degree of reorganization of cellular matter and in more robust control over the size of the growing vascular network.

PACS number(s): 87.18.Hf, 87.10.+e, 87.17.+d

I. INTRODUCTION

Biological systems are characterized by large numbers of functionally similar elements that interact selectively and nonlinearly to produce a complex behavior. These systems often show elaborate hierarchies of feedback regulation [1,2]. Feedback mechanisms are often based on both attractive and repulsive signaling molecules.

The need for repulsive factors in cellular aggregation processes has been known for a long time [3]. The presence of these factors along with attractive ones produces instabilities that drive the process of pattern formation with morphological characteristics determined by the rates of the chemical reactions and diffusion.

In vascularization dynamics, the role played by repulsive factors is a subtle one. Recent research has shown that repulsive factors are required to endow the biological system with the necessary degree of plasticity and robustness, allowing a graded control of cellular migration rate and redirectioning during migration [4].

Cellular aggregation is central in blood vessel formation, a process that has been thoroughly studied experimentally for its fundamental relevance in many physiological and pathological conditions [5]. Experimental assays of the process have been devised which allow a high degree of reproducibility and control of parameters. Biological insight obtained this way has led to the formulation of a concise mathematical model that explains the main dynamical and geometrical properties of experimental vascular network formation [6]. The good agreement of the model predictions with observations provides evidence that experimental capillary network formation (vasculogenesis) is driven by a cross-talk mechanism, where cells release and respond to chemoattractive soluble factors that stimulate motility [7]. Recent experiments in mice have confirmed the proposed autocrine cross-talk mechanism showing its essential role in in vivo angiogenesis [8]. Since chemotactric factors have a finite lifetime, this introduces in the model a typical length scale and suggests that Nature realizes this way a control on the characteristic size of capillary networks. This is confirmed by the experimental observation that the characteristic length scale introduced with the initial conditions, which is a typical cell-cell distance and is a function of the initial cell density, is disrupted by a dynamical process that rearranges the structures on a constant length scale of the order of the diffusion range of the soluble factors. Further, independent confirmation is likely to come from experiments on mice [9] where deletion of growth factors with smaller diffusivities results in larger capillary network sizes. In particular, the experimental results of Ref. [9] are strikingly reminiscent of the model simulations of Ref. [7].

Let us notice here that the existence of a control mechanism on the size of capillary networks should come as no surprise, since intercapillary distances ranging between 50 and 300 μm are required for optimal metabolic exchange [10]. As a matter of fact, the capillary networks of the vertebrates are all characterized by these characteristic dimensions.

A natural extension of the model [6,7] consists of taking into account the action of soluble repulsive factors, which are known to play a biologically relevant role. Their inclusion in the dynamical scheme should allow us to test quantitatively current ideas about their role and action. In this paper, we study the effect of this extension through analytical arguments and computer simulations and find that the action of repulsive factors results in a larger degree of reorganization of cellular matter and in more robust control over the size of the growing vascular network.

The ability to reorganize vascular networks is of peculiar relevance in living beings, e.g., during embryonic growth [11] and in the healing of wounds [12]. Our numerical simu-
lations show that starting from commonly realized experimental conditions where cells are initially randomly dispersed on a flat surface, the dynamical system generated by the diffusion of an attractive and a repulsive chemical factor allows for a larger degree of reorganization of cellular matter with respect to the situation where only an attractive factor is present. This is due to the “screening” effect realized by the repulsive factor in the cell proximity, which allows for a more even distribution of the attractive signal in space and a more efficient reorganization dynamics.

A second function performed by repulsive factors is to provide robustness and sharpness of the size control mechanism. We show that both properties are strictly related to the analytic properties of the integral kernel, which appears in the solution of the equation for the diffusion of chemical substances. This kernel can depress wavelengths that are, respectively, larger and smaller than the chemical range, acting as a band-pass filter in the spatial domain. Our analysis shows that in order to have a sharper and more robust control on the structure size, at least an attractive and a repulsive signal must be present. We expect that this prediction can be verified experimentally.

The paper is organized as follows. Section II summarizes some background knowledge on the biology of vascular network formation. In Sec. III, we introduce the model and study the analytical properties of the integral kernel that appears in the solution of the relevant diffusion equations. Section IV contains numerical results and analysis of the geometry of vascular structures. In Sec. V, we conclude discussing the possible relevance of our results for future studies. In the Appendix, we present the algorithm used for image analysis.

II. EXPERIMENTAL VASCULOGENESIS

Experiments of in vitro vascularization are an ideal test for theories of vascularization dynamics because the main parameters are under control: for instance, it is possible to perform experiments with a given cellular population, with the same values of physical parameters and with the same initial conditions, and one can track cell motion with great precision through noninvasive means such as videomicroscopy. Experiments can be repeated many times, so that statistical methods can be used to get quantitative information on the system.

In the experiments, endothelial cells are randomly dispersed on Matrigel, a surface which favors cell motility and has biochemical characteristics similar to living tissues, and observed while they autonomously organize to form a two-dimensional network and roll up to form vascular tubes [13,14] (Fig. 1). The whole process needs 12–14 h to complete. An accurate statistics of individual cell trajectories has been presented in Ref. [7], showing that in the early stages of network formation, cell motion has marked directional persistence, pointing toward zones of a higher concentrations of cells.

The capillarylike network is a collection of nodes connected by chords. The mean chord length has the approximately constant value \( l_0 \approx 200 \mu m \) over a range of values of the density \( \bar{n} \) extending from 100 to 250 cells/mm\(^2\). Corre-
understanding of the experimental results of Ref. [9]. Furthermore, it is known that isoforms of VEGF-A act in a coordinated fashion to recruit and expand tumor vasculature [22].

III. THE MODEL

A. General framework

We propose here a generalization of the model of Ref. [6] that takes into account the existence of several types of soluble factors, characterized by different diffusivities and chemical actions (attractive or repulsive). The endothelial cell population is described by a continuous distribution of density \( n \) and Eulerian velocity \( \mathbf{v} \), and we assume the presence of \( F \) families of soluble molecules, whose concentrations are denoted, respectively, by \( c_1, \ldots, c_F \).

We shall study in more detail the two-field model where \( c_A \) and \( c_R \) represent, respectively, the concentrations of attracting and repulsive factors. For the sake of simplicity, we consider a linear response of cells to the concentration of attractive or repulsive factors \( \sigma \). The model presented in Ref. [6] is then generalized to the following equations:

\[
\frac{\partial n}{\partial t} + \nabla \cdot (n \mathbf{v}) = 0, \tag{1a}
\]

\[
\frac{\partial \mathbf{v}}{\partial t} + \mathbf{v} \cdot \nabla \mathbf{v} = \nabla \sum_{k=1}^{F} \mu_k c_k - f(n) \mathbf{v}, \tag{1b}
\]

\[
\frac{\partial c_k}{\partial t} = D_k \nabla^2 c_k + \alpha_k n - \tau_k^{-1} c_k \quad (k = 1, \ldots, F), \tag{1c}
\]

where \( D_k, \alpha_k, \tau_k \) (\( k = 1, \ldots, F \)) are, respectively, the diffusion coefficient, the rate of release, and the characteristic degradation time of the \( k \)th soluble factor, and \( \mu_k \) which is assumed to be positive for attractive factors and negative for repulsive factors, measures the strength of the cell response to the concentration gradient \( \nabla c_k \). In the experiments, cells are initially randomly distributed on Matrigel. We therefore impose initial conditions in the form of a set of randomly distributed bell-shaped bumps of unitary volume having width of the order of the average cell radius, with null velocities.

Regularity of solutions and approach to a stationary state can be obtained by the addition on the right-hand side (r.h.s.) of Eq. (1b) of \( ad hoc \) phenomenological terms [6,15,23]. These terms are not really essential and just provide a clean way of mimicking the biological system by freezing the evolution when cells get close together. In this paper, we shall make use of a density-dependent linear friction term \( -f(n) \mathbf{v} \), where \( f(n) \) is zero for low densities and increases rapidly above a threshold of a few times the heights of the initial bumps [24]. The biological motivation for this term is that cells establishing reciprocal contacts are observed to progressively slow down migration, as if cell-cell contacts switched off migration in favor of the establishment of stronger binding to the gel matrix [7].

In the fast diffusion approximation, i.e., neglecting the time derivative on the left-hand side (l.h.s.) of Eq. (1c), it is possible to solve for \( c_k \) and to substitute in Eq. (1b), obtaining the simpler system

\[
\frac{\partial n}{\partial t} + \nabla \cdot (n \mathbf{v}) = 0, \tag{2a}
\]

\[
\frac{\partial \mathbf{v}}{\partial t} + \mathbf{v} \cdot \nabla \mathbf{v} = \mathcal{L} n - f(n) \mathbf{v}, \tag{2b}
\]

where \( \mathcal{L} = \sum_{k=1}^{F} \left( \mu_k \alpha_k / D_k \right) \cdot \nabla \left( r_k^2 - \nabla^2 \right)^{-1} \) and \( r_k = \sqrt{D_k \tau_k} \) is the characteristic interaction range of the \( k \)th chemical factor [25].

The dynamical behavior of system (1) is as follows. Initially, nonzero velocities are built up by the chemoattractive term due to the presence of random inhomogeneities in the density distribution. Then, Burgers’ dynamics amplifies the...
in the neighborhood of a bump of cellular matter, in the presence of a purely attractive factor $c_A$ [panel (a)] and of both attractive and repulsive factors $c_A$ and $c_R$ [panel (b)]. (a) Distribution of $c_A$ and graph of the gradient field $\nabla c_A$. (b) Distribution of $c_A-c_R$ and graph of the gradient field $\nabla(c_A-c_R)$.

characterized by the asymptotic behavior $|L_k| \sim 1/k^3$, corresponding to a sharper bandwidth and a higher selectivity. For intermediate values $\sigma \approx 1$, one observes a crossover for $k \sim 1/\sqrt{1-\sigma}$ to the $\sigma=0$ behavior.

The control properties of the transfer function $L_k$ influence the geometry of the network structures formed by the evolution of Eq. (1) from random initial conditions. Initially, cells are seeded in random positions. This introduces in the model the length scale $r_{c.c}=L/\sqrt{N}$, where $L$ is the surface size and $N$ the cells number, which is the typical cell-cell distance. This length scale competes with $r_A$. For the physiologically interesting values of $N (N \approx 100$ for $L=1$ mm), one has $r_{c.c} < r_A$. Dynamical evolution, controlled by the action of the operator $L$, can destroy this scale and rearrange matter in a network of typical length scale $r_A$.

The presence of a repulsive factor has the effect of screening high concentrations of activator in the cell proximity. In Fig. 3, the vector field $\nabla n$ is plotted for $\sigma=0$ and $1$. In the second case (perfect balance of repulsive and attractive ac-
Repulsive action results in larger reorganization of cellular matter, starting from the same initial conditions: (a) Final point of dynamical evolution in the presence of a purely attractive factor ($c_R=0$). (b) Final point of dynamical evolution in the presence of both attractive and repulsive factors ($\sigma=1$). Repulsive action results in a larger redistribution of cellular matter with respect to case (a). (c) Graph showing the concentration of attractive factor $c_A$ for (a). Arrows indicate the gradient of $c_A$. (d) Graph showing the difference $c_A - c_R$ between the concentration of attractive and repulsive factors for (b). Arrows indicate the gradient of $c_A - c_R$. Simulations were performed using $r_A = 300 \, \mu m$, $r_R = 150 \, \mu m$, and $\bar{n} = 175$ cells/mm$^2$ on a square lattice of $256 \times 256$ nodes.
Maximal reorganization efficiency is obtained when the attractive/H11003 of 256/H20849 results were obtained by 24 simulations of Eqs. H20849 efficiency is observed in the presence of repulsive factors/H20855 linear dependence of/H20849...
The results were obtained by 24 simulations of Eqs. (1) for each value of $r_A$ letting $r_R=r_A/2$ and $\bar{n}=175$ cells/mm$^2$ using a square lattice of $256 \times 256$ nodes. The three-dimensional diffusion equation (1c) was solved using a cubic lattice of $256 \times 256 \times 64$ nodes. Dotted lines are intended as guides for the eye.

length scales of observed structures can be obtained only if an attractive and a repulsive factor are present and $\sigma = 1$.

IV. NUMERICAL RESULTS AND GEOMETRY OF THE NETWORK

In order to verify the validity of previous analytical arguments, we performed extensive numerical simulations of model (1) with two families of soluble molecules and values of the ratio $\sigma$ varying between 0—no repulsive factors—and 1—perfect balance of attractive and repulsive factors in the cell proximity [32].

Simulations performed in the presence or absence of repulsive action show the qualitative fact that repulsive dynamics results in a larger reorganization of cellular matter (Fig. 4). A quantitative measure of this effect is the mean square displacement of cellular matter (Fig. 5). A better reorganization process requires that the distribution of cellular matter is reorganized on larger structures and, therefore, that cells travel over longer distances. Figure 5 shows that when both repulsion and attraction are present, the mean square displacement of cells is larger compared to the case in which only an attracting factor is present. This results in larger network structures and thicker chords (Fig. 4).

The degree of dynamical reorganization of network structures by attractive and repulsive factors is shown by the dependence of the network statistical geometry on the interaction radii $r_A$, $r_R$. Efficient reorganization implies that geometrical properties of the network such as the average chord length are strictly regulated by the characteristic interaction radii $r_A$, $r_R$ of the attractive and repulsive factors, and not by the characteristic length determined by the initial conditions. This length scale can in fact be destroyed by the dynamical reorganization process, which reorganizes cellular matter in a network of typical length $r_A$. Correspondingly, a robust control results in the statistical geometry not depending on the initial density $\bar{n}$. Such a robust control was observed in [7], where it was shown that the mean chord length is independent of the density $\bar{n}$ over a range of values extending from 100 to 250 cells/mm$^2$.

To investigate how the interaction radii $r_A$, $r_R$ of the attractive and repulsive factors control the geometrical properties of the observed vascular structures, we have measured the chord length distribution resulting from a large number of random realizations of the initial conditions, varying $r_A$ for fixed $r_A/r_R$ [33,34] (the chord length distribution has been measured by the algorithm described in the Appendix). To study the robustness of the geometrical control, we have also studied how the average chord length varies changing the average density $\bar{n}$ for fixed $r_A$, $r_R$. 
is realized only when attractive and repulsive factors balance in the cells proximity. In this case ($\sigma=1$), the structure size is determined by $r_A$ and control on the typical length scale is robust, since $\langle l \rangle$ is almost independent of the initial cells density.

In standard experimental conditions $[7, 18]$ soluble factors actually diffuse in 3D in the fluid medium, while cell movement is confined to the 2D Matrigel surface, corresponding to the dimensionally hybrid situation described by our simulations. In these conditions, it has been experimentally checked that the average chord length of observed networks does not depend on the initial cell density $[7, 15]$, i.e., on $r_{i.c.}$. Comparison with the results of the simulations (Fig. 9), where dependence on $r_A$ is actually observed only when the attractive action is balanced by repulsion on scales of the order of the cell size, suggests that repulsive factors are actually playing a role in determining the size of patterns in experimental vasculogenesis.

V. CONCLUSIONS

We presented a model for in vitro vascularization experiments, which generalizes the model proposed in Ref. $[6]$ in order to take into account the presence of more families of soluble chemotacting factors, and in particular the presence of repulsive factors. The model allows us to discuss the question of geometrical robustness in the process of vascular network formation, which is physiologically relevant, since the size of capillaries is strictly related to their functionality. Recent experiments $[4]$ have shown that the role of repulsive factors is essential to ensure the necessary degree of plasticity of vascular networks. They allow a graded control of cellular migration rate and redirectioning during migration.

Our results show that the presence of repulsive factors allows a better reorganization process and a better control on the size of the resulting patterns. From a mathematical point of view, the main geometrical properties of the observed networks are encoded in the integral operator which solves the equation of diffusion for the chemical factors. In particular geometries, for example in dimensionally hybrid situations similar to the ones realized in in vitro experiments, reorganization of the initial configuration and robust control on the final configuration size can be ensured only in the presence of at least an attractive and a repulsive factor, whose actions balance in the cell proximity.

We expect that this theoretical prediction will be the subject of future experimental studies, focusing on the question of how control on the geometrical properties of vascular networks is realized and which role repulsive factors play in the morphogenetic process.

Another area where our model could be tested is the study of vascularization in living beings $[9]$. It is known that various VEGF-A isoforms with different diffusion constants (providing different length scales) exist. Removing the less diffusible forms of VEGF-A by gene targeting leads to the formation of vascular structures characterized by different length scales $[9]$, as suggested by our model. This could be another step to a more quantitative understanding of the molecular mechanisms of vascularization.
Different theoretical models for in vitro vascularization have been proposed [35–37], focusing on cellular network reorganization driven by cellular traction [38]. These models were initially motivated by experiments performed with bovine aortic endothelial cells (BAECs) [35,38]. Both our model and this class of models are mathematical idealizations of reality, obtained in different physical/biological limits. Our model is obtained in the limit in which mechanic effects are negligible and cell motion is mainly directed, while models of Refs. [35–37] are obtained in the limit in which mechanic effects are dominant. Both classes of models reproduce well the vascular patterning and different experimental observations and therefore neither of them seems currently complete. We believe, however, that the physical/biological limits in which the models are obtained may be realizable under different laboratory conditions. In the assays described in Ref. [7], directed, amoeboid cell motion is clearly observed [39], autocrine production of VEGF-A is also directly observed, and stress-induced deformation of the matrix substrate does not appear in the first hours of observation, but only toward the end of the vasculogenic process, which, as is explicitly stated in Ref. [7], is not described by our model. Instead, in the assays described in Ref. [38] and modeled in Ref. [35], stress-induced deformations of the gel matrix are observed from the very beginning. We argue that the difference between the two experiments lies both in the different cell density and in the use of different endothelial cell (EC) populations in the two cases: human ECs from umbilical vein (HUVEC) and adrenal cortex versus bovine ECs from aorta (BAEC). In any case, the predominance of amoeboid directed motion in our models (HUVEC and hu-

FIG. 11. (a) Two-level image obtained through numerical simulation, for diffusion in a three-dimensional environment with $r_A = 400 \text{ m}, r_B = 200 \text{ m},$ and $\sigma = 1.$ (b) Skeleton obtained by applying the proposed thinning procedure. (c) Representation as a formal graph. (d) Formal graph after pruning step.

man adrenal cortex ECs) and of motion along stress-induced deformations (mesenchymal migration) in Vernon’s experiments with BAEC is clear from the photographs of the experiments. Ameboid and mesenchymal migration can be considered as different programs that an individual cell is able to execute (for a distinction between amoeboid and mesenchymal migration, see, for example, Ref. [40]). We would also like to stress that network formation would be observed in our model even without chemotaxis [29,30]. What chemotaxis adds to the picture is a plausible means of controlling the network characteristic scale. This mechanism could in principle be quite sophisticated, as explained in the present paper. Our group [7] and others [8,16–19] have shown the presence and the possible function of autocrine VEGF-A in ECs during vascular network formation both in vitro and in vivo. We hope that our present work encourages a more complete and direct analysis of the function and the dynamics of chemotactic and chemorepulsive molecules during in vitro vasculogenesis.

Finally, we observe that cell-centered models [41,42] derived from the model of Ref. [6] have recently confirmed the main results of Refs. [6,7]. These models compared with continuous models have the advantage that different physicochemical properties can be assigned to each cell and their effects on the vascular pattern can be easily studied. These individual cell models should be easily extended to reproduce the structure of the generalized model presented in this paper.

ACKNOWLEDGMENTS

We acknowledge useful discussions with M. Graziano (Politecnico di Torino). This work was supported by MURST.
“Cofin 2001,” “Cofin2003,” and Telethon-Italy Grant No. GGP04127 (to G.S.).

**APPENDIX: IMAGE ANALYSIS**

In this appendix, we present an unsupervised analysis system enabling fully automated processing of two-level images obtained through numerical simulations. The overall methodology can be broken up into several consecutive steps that lead to the generation of a graph-based representation of the blood vessel network, which allows us to evaluate effectively and accurately network-related parameters.

A skeletonization procedure is first applied on simulation images [see Fig. 10(a) and 11(a), which are representatives of simulations performed with $r_A=100$ and $400 \mu m$, respectively] in order to extract a thinned image conveying essential structural information, thus simplifying the measurement task. Skeletonization is defined as the act of identifying those pixels belonging to an object that are essential for communicating the object’s shape, preserving its topological properties [43]. The skeletonization step has been deployed using a thinning-based approach. The majority of thinning algorithms are based on a repeated stripping away of layers of pixels until no more layers can be removed. There is a set of rules defining which pixels may be removed, and frequently some sort of template-matching scheme is used to implement these rules. In this work, we selected the well-established Zhang-Suen implementation [44], which is capable of producing a sufficiently accurate skeleton. Nevertheless, there exists some problems with thinning algorithms that show up after pruning, respectively. Finally, a minimum closed-circuits discovery procedure is applied on the graph in order to identify those edges depicting a closed structure within the image. The adjacency list maintaining the ordered list of skeleton pixels constituting each edge of the undirected graph representing the vessel network together with the closed-circuits list have then been used to measure capillary chords length.

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[24] Different functional forms for $f(n)$ have been tested yielding very similar results. The simulations have been performed with $f(n) = 0.5 + \arctan[a^*(n-n_0)]/\pi$, $a = 100$, $n_0 = 8$.

[25] The finite velocity of diffusion renormalizes these scales by factors of order 1. Let us consider, for instance, the evolution of the chemical concentration $c(x,t)$ in one spatial dimension and in the case of the simple source term $n(x,t)=f(x-vt)+f(x+vt)$, representing two cells that move away from each other with velocity $v$. Equation (1c) becomes in Fourier space

$$-(i\omega+Dk^2+\tau^{-1})c_{k,a} = f(k)(\delta(\omega-kv)+\delta(\omega+kv)).$$

Solving this equation and inverting the FT, the dynamics is seen to renormalize the characteristic length scales $r_c$. The effect depends on the characteristic velocities of the system, and becomes relevant when the system's typical velocity $v$ becomes $v \gg \sqrt{D/\tau}$. The experiments show that the characteristic cell velocity is $v \sim \sqrt{D/\tau}$, so the renormalization factor is of order 1 and the fast diffusion approximation is qualitatively correct.

[26] To maintain the substantially attractive mechanism of cell communication, we shall assume that $\sigma \approx 1$.


[31] Since the mass of soluble factors is $\sim 200$ kDa and experiments are performed at $T = 37$ °C, weight becomes relevant at a height $h \sim 1$ m. Petri dishes are filled with $\sim 0.5$ cm of liquid and we can assume that soluble molecules are freely diffusing in 3D over the 4 mm$^2$ observed surface.

[32] The numerical simulations were performed on square boxes of size $L = 2$ mm with periodic boundary conditions, using a finite volume method. We used the experimental values for $D$ and $\tau$, while the unknown parameters $\mu, \alpha$ were set to 1 (which amounts to appropriate rescaling of time and the concentration field $c$). The initial conditions were given by throwing the same number of cells as in the biological experiments in random positions inside the box, with zero velocities and zero concentration of the soluble factor, with a single cell given initially by a Gaussian bump of width $\sigma = 30$ µm and unitary weight in the integrated cell density field $n$. Starting from these conditions, Eqs. (1) were numerically integrated. The simulation was stopped when the vascular network was formed, or a stationary state was reached. In the figures, black areas represent regions filled with cells, that is, regions where the density exceeds a given threshold; white areas represent the underlying substrate. The threshold was chosen to coincide with the close-packing density $\sim 1/(2\pi\sigma^2)$.


[39] See the supplementary on-line movie of Ref. [7], http://www.nature.com/embold/journal/v22/n8/extref/759205a1.txt.


[43] The precise definition of the skeleton of a region $R$ with border $B$ is the following: for each $p$ in $R$, find its closest neighbor in $B$. If $p$ has more than one such closest neighbor, then $p$ belongs to the skeleton of $R$.


