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Systems Biology of Vascular Calcification

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Abstract

Vascular calcification, a prevalent and progressive disorder, involves numerous interactive, autocrine, paracrine, and endocrine regulatory mechanisms and is thus ideally suited for analysis using a systems approach. This approach focuses on creating quantitative, testable models of complex biological systems that take into consideration the time dimension. They are usually expressed as mathematical models, and because of their time dependence and complexity, they usually require computer simulation to determine predicted outcomes. Here, we provide an example of such a model used to analyze self-organization and mineralization of vascular stem cells, using partial differential equations capable of accurately predicting experimental outcomes. Such systems-based models are useful in many aspects of cardiovascular medicine.

Vascular calcification is prevalent and progressive in cardiovascular disease, diabetes, and chronic kidney disease, and it directly contributes to cardiovascular morbidity and mortality (Demer and Tintut 2008, Hamirani et al. 2008, London et al. 2008). Over the past decade, it has become recognized as an actively regulated process, and the nature of the cells and molecular events that produce the hydroxyapatite mineral are under intense investigation (Morony et al. 2008, Shroff et al. 2008, Speer et al. 2009). In atherosclerotic plaque, some cells appear to undergo osteogenic and/or chondrogenic differentiation, forming bone and cartilage-like matrix and, with angiogenic invasion, forming mature bone with marrow.

Biomineralization also occurs often in the extracellular matrix of cardiac valves, microvessels, and in the medial layer of large arteries without atherosclerosis. Because the process involves interactive organ, tissue, and cellular regulatory systems with feedback control, it is ideally suited for analysis by a systems approach.

Regulatory Complexity of Vascular Calcification

The regulatory factors and endocrine systems that control vascular calcification (Table 1) are interdependent and complex. Many of the same stimuli that affect vascular calcification have opposite effects on skeletal bone, and release of factors from skeletal bone, in turn, affects vascular calcification, thereby emphasizing the need for consideration of multiple organ

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systems for accurate predictions. As another example of the complexity of the system, the kidney serves as a central integrator for many, including the vitamin D, phosphate, and parathyroid systems. Indeed, as noted by Towler, end-stage renal disease is a “perfect storm” for vascular calcification (Vattikuti and Towler 2004). As one example, fibroblast growth factor-23 (FGF-23) regulates serum phosphate homeostasis through multiple organs (Figure 1). When serum phosphate is elevated, FGF-23 expression is induced in skeletal osteoblasts and osteocytes and is released to the circulation, where it lowers serum phosphate both directly and indirectly. It directly inhibits renal phosphate reabsorption by suppressing expression of sodium-phosphate cotransporters in the kidney. It indirectly inhibits intestinal phosphate absorption by reducing serum 1,25 (OH)₂- vitamin D levels by inhibiting renal expression of 25-hydroxyD-1 α -hydroxylase, which activates vitamin D, and enhances expression of 25-hydroxyD-24-hydroxylase, which converts vitamin D to a less active form. In conditions of nonfunctional or deficient FGF-23, elevated serum 1,25(OH)₂D and phosphate result in vascular and soft tissue calcification (Inaba et al. 2006, Shimada et al. 2004). Adding further complexity with a secondary negative feedback loop, FGF-23 appears to directly inhibit parathyroid hormone secretion (Ben-Dov et al. 2007), which reduces bone resorption and, hence, phosphate levels. This one example underscores the need for the systems approach and mathematical modeling to predict outcomes.

Systems Biology

Although widely viewed as *avant garde*, the term “systems biology” is probably derived from the field of “systems analysis” within the discipline of “systems engineering,” which can be traced back to Bell Laboratories in the 1940s, where it was used to identify and manipulate the properties of complex control mechanisms. In biology, as in engineering, the systems approach is needed because behavior of complex organisms may differ greatly from the sum of the properties of individual molecules or parts due to interactions (Tegner et al. 2007). Leading away from reductionism, the systems approach incorporates rigorous quantitative data and knowledge of multiscale, dynamic (time-dependent) behavior to characterize the interactions. This approach was used in cardiovascular physiology decades ago (Sagawa 1972). It may be applied to entire populations, organisms, organ systems, cells, or molecules.

The difference between systems and conventional biology approaches is evident in their schematic depictions of predicted mechanisms. In conventional biology, hypothesized mechanisms are typically depicted as static “boxes-and-arrows” diagrams showing components (such as signaling molecules, gene expression, regulatory factors) connected by either inductive or inhibitory arrows indicating either positive or negative effects. Neither time-dependent information (dynamic) nor quantitative information can be discerned so that these diagrams generally lack the capacity for prediction or testing (Isalan and Morrison 2009). Indeed, where delayed feedback loops are present, the net effects on downstream factors could range from strong induction to strong inhibition. In contrast, in the systems approach, hypothesized mechanisms are depicted as mathematical functions equations or control diagrams that provide explicit time dependence and specific, quantitative information on the strength of predicted interactions. The mathematical functions in these models specify the exact relationships between variables and changes with time, allowing the models to generate predictions that can be tested experimentally, as in the model of cardiovascular physiology developed by Arthur Guyton 30 years ago (Sagawa 1975) (Figure 2).

Because biological interactions are typically time dependent, their analysis (ie, outcome predictions) generally requires development of a mathematical model of the interactions, usually a differential equation describing the changes in characteristics over time. Because biological interactions are also typically nonlinear, the mathematical models are complex enough to require computer simulation. Thus, the term “systems biology” is often used in

conjunction with the terms “computational biology” and/or “computational modeling.” However, computer simulation is simply one approach (usually the only practical one) to solving the model equations for specified conditions. Computational biology can refer to (1) the use of computer technology to simulate differential equations representing the fundamental physiological processes or (2) the use of computer technology to find statistical correlations in data sets from high-throughput genomic, proteomic, and/or metabolomic technologies. In this review, we will focus only on the former.

Mathematical models define the exact nature of the hypothesized interaction and allow testing of predictions. When the mathematical model is insufficiently detailed, it can be refined by iterative approaches comparing results with predictions to achieve a more accurate model. Mathematical models are required once the number of interacting systems becomes too great to do calculations on the back of an envelope. Importantly, computer simulations, also known as *in silico* models, are not simply animations. Instead, they simulate a wide range of outcomes in response to changes in parameters. They allow the user to select values for biological parameters and obtain predictions of the outcomes. One reason mathematical models are not more widely used is that values for many biological parameters (the constants in the equations) may be difficult or sometimes impossible to determine accurately.

Mathematical models are adjustable, like a microscope, to different levels of detail resolution depending on the question. If a mathematical model is too simple to address a question, it can be refined to add more realistic complexity, just as the microscope can be adjusted to a different power, depending on the question. As another analogy, a restaurant’s dessert selection may be modeled at different levels of complexity such as a paper menu or a tray of models. What is important is that the user knows the model is not intended to duplicate reality; we do not eat the menu.

Reaction-Diffusion Modeling of Vascular Calcification

As an example, systems analysis may be applied to vascular calcification, specifically *in vitro* vascular cell calcification. As first demonstrated in microvascular pericytes, certain types of vascular cells have the capacity to mineralize their extracellular matrix *in vitro* (Collett and Canfield 2005, Shao et al. 2007). These also have multilineage potential similar to that of mesenchymal stem cells, expressing osteogenic, chondrogenic, leiomyogenic, and marrow stromogenic differentiation factors as well as self-renewal capacity (Cheng et al. 2003, Farrington-Rock et al. 2004, Kirton et al. 2007, Tintut et al. 2003).

Over time in culture, calcifying vascular cells swirl into local alignment, then aggregate locally to form raised, multicellular nodules or ridges, approximately 100 to 500 μm in diameter and about 200 μm in height. The nodules consist of a surface of flattened, living cells over a core of extracellular matrix and apoptotic cells. The internodule spacing is about 800 μm throughout the culture. Based on their histological and ultrastructural features, we speculate that the formation of calcified nodules by these cells *in vitro* recapitulates the *in vivo* formation of calcified nodules within stenotic aortic valves and within atherosclerotic plaque.

With specific perturbations of the culture conditions, the cells self-organize into other patterns, such as parallel ridges forming labyrinthine patterns similar to zebra stripes. Unexpectedly, upon treatment with warfarin, an inhibitor of matrix GLA (γ -carboxyglutamic acid) protein (MGP), calcifying vascular cell cultures that ordinarily produce aggregates in a stripe pattern, produced stripes of double density. This complex self-organization behavior is difficult to explain without use of a mathematical model, as discussed below.

At the molecular level, a potent osteogenic differentiation factor, bone morphogenetic protein-2 (BMP-2), is expressed in these cells *in vitro* as well as in calcified human atherosclerotic plaque

(Bostrom et al. 1993). Interestingly, MGP, an unusually small molecule expressed in atherosclerotic plaque (Shanahan et al. 1994) and vascular cell culture (Tintut et al. 1998), inhibits BMP-2 (Zebboudj et al. 2002). Bone morphogenetic protein-2, a member of the transforming growth factor- β superfamily, is a recognized embryonic morphogen (as is its *Drosophila* homolog, decapentaplegic). It governs body and tissue organization during development through diffusion gradients. Although gradients of single morphogens can produce graded features, such as diminishing pigmentation, or bipolar interfaces, two morphogens are required to produce repetitive patterns, like the stepping stones of vertebral bodies, and they interact in a more complex manner such as reaction-diffusion.

A mathematical model was developed to study self-organization and differentiation of vascular cells in patterns of calcified aggregates. Reaction-Diffusion morphogenesis, a mechanism first proposed in the premolecular era (Turing 1952), yields repetitive patterns simply from diffusion gradients of two interacting morphogens, one activator and one inhibitor. This mechanism has been used to explain the many patterns of stripes and spots found in animal epithelia (Murray 1982). The phenomenon is described by a system of partial differential equations governing the change of activity over time of the “activator” and “inhibitor” morphogens, whose activity or concentration is represented by the variables U and V :

$$\begin{aligned}\frac{\partial U}{\partial t} &= D_U (\nabla^2 U) + F(U, V) \\ \frac{\partial V}{\partial t} &= D_V (\nabla^2 V) + G(U, V)\end{aligned}$$

The terms on the left side of each equation are the partial derivatives, which represent the change in activity of U and V over time. The first term of the right sides of the equations are standard spatial diffusion terms. D_U and D_V are the diffusion coefficients for the two morphogens. In general, the second terms of the right sides of the equations describe the following conditions:

- U is autocatalytic (positive feedback condition)
- U induces V , and V inhibits U (negative feedback condition)
- Both U and V are degraded with time.

For our culture system, we used the following forms (Garfinkel et al. 2004):

$$\begin{aligned}\frac{\partial U}{\partial t} &= D_U (\nabla^2 U) + \gamma \left[\frac{U^2}{(1+kU^2)V} - cU \right] \\ \frac{\partial V}{\partial t} &= D_V (\nabla^2 V) + \gamma (U^2 - eV + S)\end{aligned}$$

The constant terms represent biophysical parameters, such as exogenously administered inhibitor (S), degradation rates, diffusion coefficients, autocatalytic saturation, and the sensitivity of the morphogens to one another and themselves (comparable to the gain of the positive and negative feedback loops). Their values are specific to the morphogens of interest. Under specific conditions, when the inhibitory morphogen diffuses about 200-fold more rapidly than the activator morphogen and a small, random, deviation from uniformity arises in the culture, instability results, and the activities unfold into distributed patterns. These patterns are not normally visible unless the activity generates a cellular response, which can be imaged, such as chemotaxis, in which case the cells reveal the pattern of chemicals the way ants reveal a pattern drawn in honey.

For most putative biological reaction-diffusion systems, the morphogens have not been identified. In the case of vascular stem cells, the morphogens appear to be BMP and MGP. Cellular aggregation and matrix mineralization are known activities of BMP-2. Matrix GLA

protein is sufficiently small to achieve the high diffusion ratio with BMP-2 necessary for diffusion-reaction patterning. Using estimates of diffusion parameters for these proteins from the literature and assuming no-flux boundary conditions, as would be found in a static culture dish (disregarding gas exchange), the mathematical model was simulated computationally. The resulting patterns, displayed as black on white diagrams representing the culture domain based on a threshold value of activator activity, corresponded to the patterns produced by aggregating and mineralizing cells (Figure 3).

Predictive Value of the Math Model

The value of a mathematical model is its ability to predict results of interventions, that is, to generate hypotheses that can be tested experimentally. One prediction that emerged from this model is that addition of exogenous inhibitor (*S*) would convert the stripe pattern to a spot pattern. This prediction was confirmed experimentally by an investigator blinded to the results of the simulation: cultures treated with exogenous MGP developed a spotted pattern of nodules. A second prediction that emerged from the model is that addition of an inhibitor (warfarin) of the inhibitory morphogen (MGP) would cause a doubling of the frequency of stripes in the labyrinthine pattern. This prediction had already been observed experimentally, before the mathematical model was developed. We speculate that these patterns generated in the mathematical and in vitro models may explain why atherosclerotic plaques appear in a heterogeneous pattern within vessels. Interestingly, warfarin use in pregnancy leads to a punctate skeletal deformity in the fetus (Pauli 1988). The beauty of such mathematical models is that additional factors or processes can be incorporated simply by modifying the constants in the equations or by introducing new terms into the equations, empowering the model to investigate systems over a wide range of complexity.

Conclusion

Regulatory factors from multiple organs and tissues contribute to vascular calcification through dynamic processes involving complex interactions and multiple feedback regulatory mechanisms. In culture, vascular cells self-organize and mineralize via a reaction-diffusion process through a mechanism that can be analyzed and understood only through a mathematical model. This approach may be useful for investigating other complex, feedback-regulated, biological, and pathological processes.

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Figure 1.

Interactions among several organs and secreted factors that influence vascular calcification (not fully inclusive). *Arrows* depict some of the effects of one regulatory factor, FGF-23. *Solid arrows* represent direct and *dashed arrows* represent indirect effects. PTH indicates parathyroid hormone; IGF, insulin-like growth factor; RANKL, receptor activator of nuclear factor-kappa B ligand; OPG, osteoprotegerin; TNF, tumor necrosis factor; IL, interleukin.

Figure 2.

An example of the use of a control diagram to depict quantifiable interactions in a complex system, here cardiovascular hemodynamics as modeled by Guyton et al. The *arrows* represent input variables, in this case, cardiovascular regulatory factors, such as arterial pressure (AP), urine output (UO), extracellular fluid volume (ECFV), blood volume (BV), mean systemic filling pressure (MSP), right atrial pressure (RAP), and so on. This figure is reproduced with kind permission from Springer Science+Business Media: Annals of Biomedical Engineering, Critique of a Large-Scale Organ System Model: Guytonian Cardiovascular Model, 3, 1975, 396, K. Sagawa, figure 10, copyright 1975 by Academic Press, Inc.

Figure 3. Pattern of three-dimensional structures formed by calcifying vascular cells in vitro (*right panel*) and the pattern predicted by a math model (*left panel*) based on the reaction-diffusion interactions between an activating morphogen, BMP-2, and an inhibitor, MGP.

Table 1

Partial list of regulatory factors in vascular calcification

Inhibitory	Stimulatory
Adiponectin	Apoptosis
Axl	BMP-2
Fetuin	Calcium
Fibrillin	Chronic hyperparathyroidism
High-density lipoprotein	Collagen I
Insulin	Estradiol
Insulin-like growth factor	FGF-23
MGP	Inflammatory cytokines
Omega-3 fatty acids	Inorganic phosphate
Osteocalcin	Leptin
Osteoprotegerin	Mechanical stretch
Osteopontin	Msx-2
Parathyroid (intermittent application)	Oxidative stress
Pyrophosphate	Oxidized lipids
Statins	Protein Kinase A activators
Warfarin	Receptor activator of NF- κ B ligand Runx2 Transforming growth factor- β Vitamin D Wnt/Low-density lipoprotein receptor related protein 5