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Abstract

The completion of the Human Genome Project and ongoing sequencing of mouse, rat and other genomes has led to an explosion of genetics-related technologies that are finding their way into all areas of biological research; the field of biorheology is no exception. Here we outline how two disparate modern molecular techniques, microarray analyses of gene expression and real-time spatial imaging of living cell structures, are being utilized in studies of endothelial mechanotransduction associated with controlled shear stress *in vitro* and haemodynamics *in vivo*. We emphasize the value of such techniques as components of an integrated understanding of vascular rheology. In mechanotransduction, a systems approach is recommended that encompasses fluid dynamics, cell biomechanics, live cell imaging, and the biochemical, cell biology and molecular biology methods that now encompass genomics. Microarrays are a useful and powerful tool for such integration by identifying simultaneous changes in the expression of many genes associated with interconnecting mechanoresponsive cellular pathways.

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The convergence of haemodynamics, genomics, and endothelial structure in studies of the focal origin of atherosclerosis [1]

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Abstract. The completion of the Human Genome Project and ongoing sequencing of mouse, rat and other genomes has led to an explosion of genetics-related technologies that are finding their way into all areas of biological research; the field of biorheology is no exception. Here we outline how two disparate modern molecular techniques, microarray analyses of gene expression and real-time spatial imaging of living cell structures, are being utilized in studies of endothelial mechanotransduction associated with controlled shear stress *in vitro* and haemodynamics *in vivo*. We emphasize the value of such techniques as components of an integrated understanding of vascular rheology. In mechanotransduction, a systems approach is recommended that encompasses fluid dynamics, cell biomechanics, live cell imaging, and the biochemical, cell biology and molecular biology methods that now encompass genomics. Microarrays are a useful and powerful tool for such integration by identifying simultaneous changes in the expression of many genes associated with interconnecting mechanoresponsive cellular pathways.

1. Introduction

Over the past two decades an appreciation of the importance of the mechanical environment of cells in determining many aspects of their physiology and pathology has emerged. This is especially true in vascular pathophysiology where the relationships between haemodynamic forces and the arterial endothelium are under intense study [4,7,28]. Quantitative vascular biology has been stimulated by the rediscovery, at a mechanistic level, of physiological flow-mediated vasoregulation [16,27] and by renewed interest in haemodynamics as the determinant of regional and focal localization of atherosclerotic lesions [29]. Driven principally by the development of sophisticated technologies in cell biology, biochemistry and imaging, such studies are now accelerated by the introduction of genomic techniques that allow a level of systems integration previously unattainable. Here we outline how these new techniques can address the relationships among haemodynamics, the endothelium, and the location of early atherosclerotic lesions [8,11]. The new methodologies extend the classic fluid dynamic studies and biological concepts developed by Caro and colleagues at Imperial College, London [3], and Fry, Roach, and

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others in the U.S. and Canada [5,15] and enhance the earlier work by providing molecular insights into cellular responses associated with such complex biomechanical systems.

2. Length scales

The focal origin of atherosclerosis and its localization to predictable regions of the arterial tree are strongly correlated with spatially complex haemodynamics that include flow separation/reattachment and flow instabilities [3,18,23]. Non-aligned endothelial cell shape and altered gene and protein expression are localized in the arterial tree to regions of low average shear stress but high gradients of shear stresses; these locations include branches, bifurcations, and regions of vessel curvature. The distribution of forces acting at the haemodynamic interface, the endothelium, can be measured over regions of many cells where the flow characteristics are relatively uniform, or at the level of smaller cell groupings where flow separations, haemodynamic disturbances and sharp gradients of shear stress are prevalent (referred to as "disturbed" flow regions). The latter add significant spatial and temporal components to the analyses. Single cell imaging *in situ* [26] and subcellular imaging *in vitro* [19,20] further broaden the scale range. Mechanotransduction in the endothelium can therefore be considered over multiple length scales ranging from centimeters of arterial surface to nanometers of subcellular separation (Fig. 1). Superimposed on the spatial scales are temporal variations occurring in intervals ranging from milliseconds to diurnal periodicities of many hours.

The haemodynamic relevance of the localization of atherosclerosis to certain portions of the arterial tree has traditionally been defined at a stage of lesion development that is later and much more extensive spatially than the events that initiated the pathological process [6,9]. It is therefore of interest to examine haemodynamics and endothelial mechanotransduction in a pre-lesional geometry and during the very earliest pathological changes. Since the disease is focal in origin, access to local and focal endothelial biology is desirable. As regions of interest within the endothelial monolayer become smaller, however, two major difficulties arise. First, investigations must analyze fewer and fewer cells, and second, additional complexities creep into the computational fluid dynamics, in part because the contribution of the geometry of the cell itself becomes significant [1,10]. There is also inherent cell-to-cell heterogeneity at the structural and biochemical levels due to differences in cell geometry and responsiveness to stimuli [10]. How can some of these obstacles be overcome?

3. A genomics approach

As outlined in Fig. 1, it is relatively straightforward to identify regions of the arterial tree predisposed to lesion development in a number of animal models. Endothelium can be removed from such regions and pooled for biochemical and genomic analyses (see below), or the cells can be directly probed and observed *in situ* by immunostaining to evaluate protein expression [26] or by *in situ* hybridization to estimate gene expression levels [13]. The two latter techniques generate good spatial information, but they are limited to the evaluation of one or two proteins or genes at a time. In contrast microarray experiments generate information about many genes simultaneously, and for some species the entire genome can be probed. Endothelial mRNAs are isolated, labeled by fluorescence or radioactivity, and hybridized to thousands of spots of cDNA or oligonucleotides, each representing a single gene (or spliced variant), immobilized on nylon filters or glass slides. The quantity of labeled RNA bound to each spot is measured by

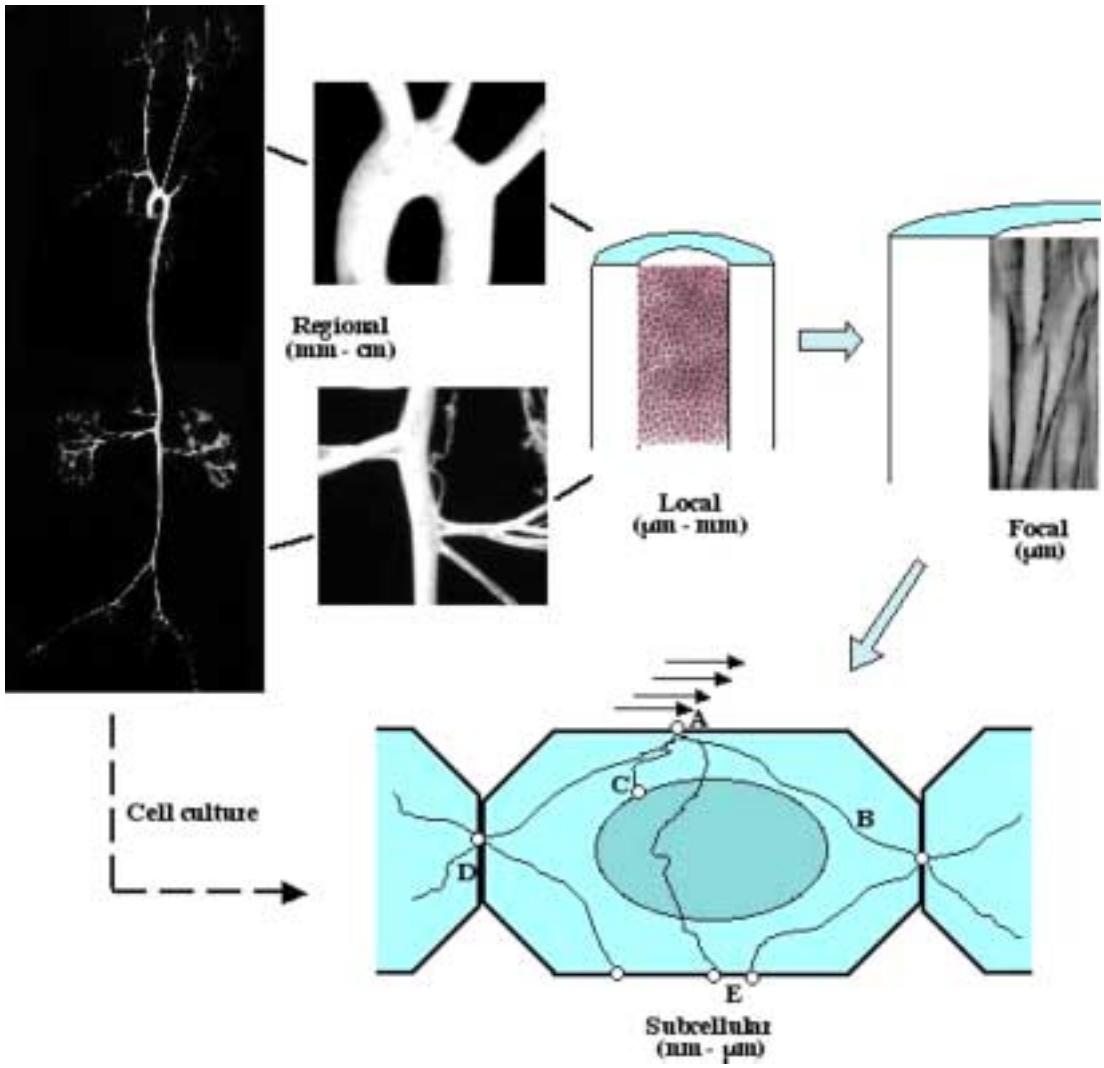

Fig. 1. Regional, local, focal, and subcellular approaches to endothelial mechanotransduction. A decentralized model of biomechanical responses is proposed at the subcellular level in which spatially-constrained physical (structural) and biochemical elements are integrated. Candidate signaling locations include the luminal cell surface (A), the cytoskeleton (B), nuclear membrane (C), intercellular junctional proteins (D), and sites of cell adhesion (E). It is proposed (see text) that genomics-based analyses will provide insights into the structures and pathways linking these elements in the coordination of biomechanical signal transduction.

the fluorescence or radioactive intensity so that the aggregate output is a snapshot of gene expression levels in the original cells. By harvesting endothelium located in different haemodynamic fields, spatially-defined gene expression profiles in the arterial tree can be compared. We have recently completed differential transcription profiling of 15,000 endothelial genes isolated from two haemodynamically-distinct regions of the pig aorta: one area susceptible to atherosclerosis and associated with disturbed flow, the other lesion-resistant and associated with undisturbed-unidirectional flow. Less than 200 genes ($\sim$1.5%) were differentially expressed between the lesion-susceptible/disturbed flow location compared with the region of directional undisturbed flow. The subset of the genome identified to be downregulated in the

endothelium of the arterial wall at lesion-prone sites may represent candidate atheroprotective genes. Conversely, upregulated endothelial genes in lesion-prone locations may be atheropermissive (Polacek et al., manuscript submitted). Similar experiments conducted in controlled flow fields *in vitro* have also recently been reported [17]. Despite the power of such a genomics approach, however, it fails to address gene expression at the level of just a few cells. The expression profiles represent the average of several million cells from a relatively large region that includes sub-regions where spatial and temporal gradients of shear stress occur over short distances [23]. If subsets of cells within the larger cell population exhibit a pro- or anti-atherosclerotic gene expression profile, their detection will be diluted by the average expression profiles of the larger population of cells. Efforts to measure multigene expression from small numbers of cells, or ideally a single cell, may circumvent this problem. In neuroscience research, Eberwine and colleagues have developed a RNA amplification method that permits expression profiles of multiple genes from individual brain cells [14]. To amplify the starting mRNA, a T7 polymerase promoter site is incorporated into each cDNA copy from the original mRNA through a dT(T7)-primed reverse-transcriptase reaction. The T7 promoter is then used to direct two rounds of linear RNA amplification by T7 polymerase, retaining much of the relative levels of mRNA expressed in the original cells. Eberwine's linear amplification strategy avoids inherent problems associated with quantitative reverse transcription polymerase chain reaction (RT-PCR), including logarithmic propagation of misincorporated bases through the amplification process and a bias towards smaller cDNA sizes [24]. Single cell RNA amplification is difficult because one cell contains only a fraction of a picogram of mRNA. Utilizing this technique, we have evaluated transcription profiles in endothelial cells isolated from different haemodynamic environments [8]. In order to improve the signal/noise and therefore the confidence limits of the technique, we are currently focusing on the analysis of small regions (fifty to several hundred endothelial cells) of artery surface and cultured monolayers.

Microarray techniques are largely a means to an end; they identify candidate genes and pathways that must be verified by other, less global techniques. It is necessary to confirm the identities and true expression levels of differentially expressed genes as revealed by microarrays. A standard check is to perform fluorescence-based real-time RT-PCR [25] on a small sample of the original mRNA used in the microarray study. Although it requires the design of unique primers for each gene, it is a rapid and very sensitive quantitative technique that allows determination of true mRNA expression levels for a manageable number of interesting genes.

The advantage of RNA expression measurements in mechanotransduction studies using microarrays is the large number of genes that can be measured simultaneously. The significance of differentially expressed genes is enhanced when they are identified as clusters known to be in the same or related biological pathways, or when suggestive of novel pathways. The genomics data then serve to identify which genes, proteins and pathways to investigate further and in greater depth using classic cell and molecular techniques. An added value, therefore, of the microarray approach is the breadth of information obtained from each experiment that may lead to new hypotheses, investigations, and interpretations.

4. Endogenous fluorescence imaging supports a decentralized model of mechanotransduction

At the other end of the scale range from genomics, studies of subcellular, structural and biochemical responses using living cells have provided mechanistic insights of endothelial mechanotransduction. Atomic force microscopy combined with finite element modeling revealed the surface topography of live endothelial cells and permitted the calculation of stress distributions at subcellular distances over the cell

surface [1]. Scanning tandem confocal microscopy demonstrated the plasticity of adhesion sites of the basal endothelial surface and their directional responses to shear stress imposed at the lumen [12]. More recently, cDNAs encoding fluorescent proteins (e.g., green fluorescent protein, GFP) have been fused to DNAs encoding cytoskeletal proteins. Newly synthesized cytoskeletal proteins are endogenously fluorescent in transfected cells. This approach has provided new insights into the real time dynamics of endothelial structure including positional displacement of the cytoskeleton in response to flow forces ([19,20], see below). These and other studies led to a decentralized model of endothelial mechanotransduction [7] that considers haemodynamic forces to not only act locally at the luminal cell surface but also to be transmitted throughout the cell via the cytoskeleton to multiple sites where conversion of mechanical forces to electrophysiological, biochemical and genetic responses may occur (Fig. 1, lower drawing). In the model, mechanotransduction results from an integrated response of multiple signaling networks that are spatially organized throughout the surfaces and interior of the cell. Since the cytoskeleton provides structural continuity between the elements, it plays a dominant role in redistributing intracellular forces [21].

Direct demonstration of the displacement and deformation of the cytoskeleton upon a step change of flow in confluent endothelial cells was investigated by time-lapse optical sectioning and deconvolution image analysis of intermediate filaments [19,20]. In collaborative studies with Dr. Robert Goldman of Northwestern University, filament dynamics were made (endogenously) visible by a GFP-vimentin fusion protein (Fig. 2A). Spatial and temporal dynamics of intermediate filaments were studied in the absence of flow, during an interval just after flow onset, and during subsequent intervals of continued flow. The collection of images of cell sections for each three-dimensional reconstruction was completed within three minutes, allowing sequential snapshots of filament position to be recorded over long periods. Volume projections of reconstructed 3D images from consecutive time points were compared for qualitative assessment of filament displacement. In the absence of flow, constitutive motion of filaments was small and random (“wriggling”), the number of filaments remained constant, and inter-filament connections were unchanged over extended periods. When a flow step (to 12 dyn/cm^2 shear stress) was imposed, however, significant directional displacement (up to 1 μm) of intermediate filaments occurred in the first interval after flow was started. Care was taken to reference and correct for any movement of the stage or glass slide upon which the cells were grown.

Filament displacements were spatially heterogeneous throughout each cell. To evaluate the images quantitatively, a correlation coefficient was computed that describes the degree of overlap of filament fluorescence between consecutive images. Significant increases in intermediate filament displacement after flow onset were measured (Fig. 2B). Further quantitative image analyses of filament displacement associated with changes of shear stress have been developed, including a 3D displacement index related to the product moment correlation coefficient as a function of time and location. Greater filament displacement above, as well as downstream of, the cell nucleus than in other parts of the cell was measured [20]. Most recently, the quantitative measurements have been extended to track as a function of time the positions of connection points between at least three filaments (“nodes”) that were extracted from skeletonized filamentous images in representative 2-dimensional optical sections. From these data, principal strains associated with the filament network throughout the cell were computed (B.P. Helmke, A. Rosen and P.F. Davies, manuscript submitted). This analysis is a direct measurement of intracellular strain using endogenous cytoskeletal elements in a physiologically-relevant mechanical environment. Through such studies, we hope to better understand the spatial (and temporal) coordination of intracellular structures and signaling molecules involved in mechanotransduction.

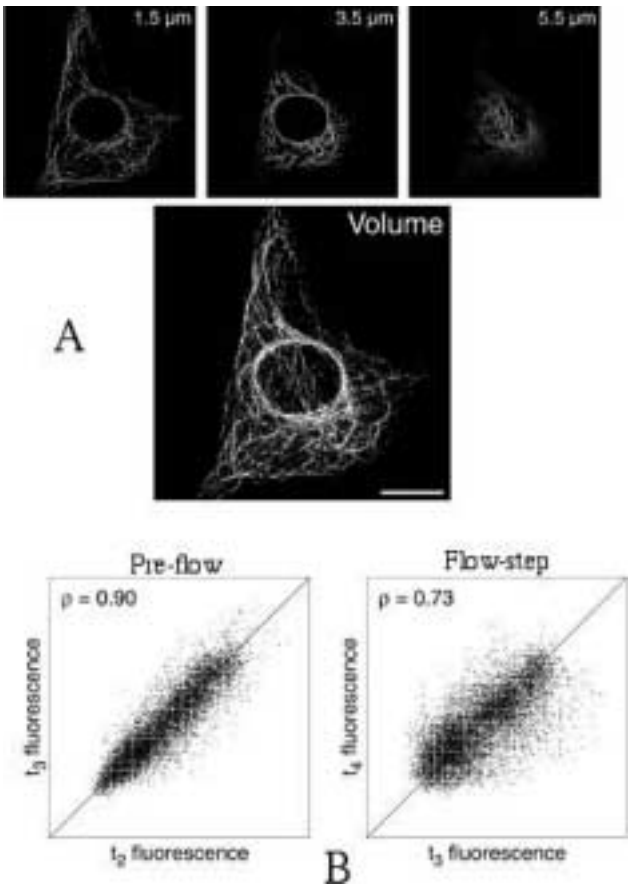

Fig. 2. A: Three-dimensional distribution of GFP-vimentin (intermediate filaments, IF) in confluent monolayers of living endothelial cells. Deconvolved optical sections at indicated heights above the coverslip (1.5, 3.5 and 5.5 μm, respectively) and a volume projection show that GFP-vimentin is distributed to the endogenous IF network in transiently expressing cells. Scale bar, 10 μm. B: Change in IF motion due to the onset of shear stress (12 dyn/cm^2). The positions of IF in fluorescence optical sections were compared at consecutive time points under no-flow conditions (t_2, t_3), and before (t_3) and after (t_4) a step increase in flow. A correlation coefficient, ρ, measured the degree of overlap of images; $\rho = 1$ for perfect overlap (no movement), and ρ decreases for less overlap (increased IF movement). A significant decrease of ρ demonstrates the marked displacement of IF position associated with the flow step. Condensed from Helmke et al., *Circ. Res.* **86** (2000), 745–752 with permission.

5. Linking the scales

The two examples above, broad based genomics on the one hand and detailed cellular image analyses on the other, appear worlds apart in scale, technique, and focus. At first glance they appear to be almost at opposite ends of the range of investigative tools that one might bring to mechanotransduction studies. However, differences in the scales of the biological approaches can be complementary in designing an integrated approach to understanding how cells and tissues sense their mechanical environment. At the subcellular level, intermediate filaments interact closely with actin microfilaments, microtubules, the cortical cytoskeleton, junctional proteins, a large number of known cytoskeletal regulatory molecules, transmembrane integrins together with their linker proteins, and the highly organized apical, basal, and junctional plasma membranes. These structural interactions are components of a complex rheological environment within the cell that provides spatial cues to modulate gene and protein expression. Cytoskele-

tal positional changes recorded over long intervals can span the time courses of short and long-term transcriptional changes. The resulting adaptation in gene and protein expression at both the single cell and tissue levels feeds back to modulate structural associations and molecular signaling interactions including detailed quantitative live cell studies using sophisticated spatial probes such as in single protein fluorescence resonance energy transfer [2] and endothelial nitric oxide detection [22].

A circle of interactions might be envisaged in which [1] microarray analyses provide the large-scale tools necessary to investigate the vast number of transcriptional and signaling pathways at the molecular scale, particularly structural interactions that provide spatial cues for molecular interactions, [2] signaling at the level of molecular interactions drives gene and protein expression, and [3] levels of gene and protein expression feed back to modulate structural associations and molecular signaling interactions. Thus, microarrays provide the large-scale analysis tools necessary to investigate the large number of pathways at the molecular scale, and the molecular scale interactions ultimately determine function at the cellular and tissue level that can be probed with microarrays.

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