Published in final edited form as:

Curr Opin Nephrol Hypertens. 2015 July ; 24(4): 393–400. doi:10.1097/MNH.0000000000000138.

Relationship of and Cross-talk Between Physical and Biologic Properties of the Glomerulus

R. Tyler Miller, M.D. and

Professor of Medicine, U.T. Southwestern Medical Center, Chief, Medical Service, Dallas VAMC, 4500 S. Lancaster Rd, Dallas, TX 75216, Tel 214-857-0409

Paul A. Janmey, Ph.D.

Professor of Physiology, Physics, and Astronomy, Institute for Medicine and Engineering, University of Pennsylvania, 1010 Vagelos Research Laboratories, 3340 Smith Walk, Philadelphia, PA 19104, 215-573-7380

R. Tyler Miller: tyler.miller@utsouthwestern.edu; Paul A. Janmey: janmey@mail.med.upenn.edu

Abstract

Purpose of review—Cells and tissues must respond to physical forces. Cells exist in an elastic environment determined by their matrix, matrix contacts, cell-cell contacts, and cytoskeletal structure. We discuss the determinants of the elastic environment of cells and its potential roles in glomerular disease.

Recent findings—Elastic environment is sufficient to induce and maintain the differentiated state of cells including myofibroblasts. New experimental techniques permit precise measurement of the elastic characteristics of normal and diseased tissues and cells, and analysis of cell behavior and cytoskeletal structure in response to mechanical and elastic stimuli. Glomeruli become soft early in the course of several disease models, yet late stages are characterized by increased stiffness and fibrosis with loss of organ function. Work in hepatic fibrosis, arterial disease, and oncology demonstrate that increased collagen crosslinking by lysyl oxidase, an early step in the diseases, can result in a sufficient increase in tissue stiffness to alter cell behavior leading to disease progression.

Summary—The elastic environment of cells and tissues provides essential signals in development, differentiation, and disease. Identifying the mechanisms that determine the elastic environment of glomerular cells will complement other approaches to reduce pathologic fibrosis and loss of tissue function.

Keywords

Glomerulus; biophysics; elasticity; fibrosis

Conflicts of Interest. The authors have no conflicts of interest.

Introduction

The roles physical environment and forces play in biologic systems can be as important as those of chemical signals. Physical factors that affect cell and tissue function include the mechanical properties (e.g. stiffness) of the environment (matrix and other cells), shear force from flowing liquids or air, stretch, or compression. The elasticity of the cellular environment represents information that elicits specific cell responses including division and differentiation. The effects of physical stimuli on cell behavior gives the mechanical environment fundamental importance in normal biology and disease.

The contribution of fibrosis in renal disease has been recognized for years, and the role of myofibroblasts in this process has become clear recently. Importantly for this review, the capacity of mechanical factors, specifically matrix elasticity, to activate tissue fibroblasts or other cells to become myofibroblasts illustrates the role of physical stimuli in biological responses [1-4]. TGF- β , a cytokine associated with increased matrix production, and fibrosis is unable to induce myofibroblast development, in the absence of stiff a matrix [5-7]. Consequently, mechanical factors are essential components of the injury and repair process, and interact with better-known chemical factors (e.g. cytokines) to mediate the balance between repair and progressive fibrosis.

Measurement and Characterization of Cell and Tissue Elastic Properties

Elastic resistance (quantified as elastic modulus, E_{Mod}) determines the degree of recoverable deformation of a substance in response to a force and is quantified in Pascals (Pa). The higher the value, the stiffer (more resistant to deformation) the substance. Elastic properties of tissues are commonly measured using macroscopic rheometry, atomic force microscopy (AFM), or micro-dindentation (similar to AFM, but larger scale). These methods either deform the tissue a set amount and measure the resulting resistance to deformation or exert a set force and measure the deformation. Such measurements produce similar values when comparing similar tissues at the same age and state of health, and demonstrate that normal tissues range from a few hundred Pa for fat and brain (very soft) to greater than 1,000 kPa for cartilage (very stiff). Normal liver is approximately 0.5 kPa, kidney is approximately 2.5 kPa, and striated muscle is approximately 10 kPa (Figure 1) [8]. The stiffness (E_{Mod}) of many soft tissues, including breast, lung, liver kidney, and some blood vessels, is in the range of 0.2 - 4 kPa, and the variance in stiffness for individual tissues types is often small, within 10-15% of the mean value [9-11].

Scars exhibit increased stiffness compared to adjacent normal tissue. This increased stiffness is sensed by the adjacent normal tissue and can lead to aberrant cell-cycle progression and abnormal function in the normal tissue as demonstrated in heart and liver [10;12;13]. The effects of scarring and matrix elasticity on the behavior of renal cells has not been studied extensively, although stretch has been investigated [14]. Differences in matrix E_{Mod} of hundreds to a few thousand Pa are sufficient to result in significant biologic effects. For example, normal breast tissue is ~ 0.3 kPa, pre-malignant breast tissue is ~ 1.2 kPa, and breast tumors are ~ 4 kPa. Normal mammary epithelial cells grown on matrix with these values take on normal, premalignant, or malignant characteristics corresponding to the

matrix E_{Mod} [15]. Discrepant values have been proposed for various tissues and tissue environments by different investigators, but the values obtained by the methods described above are likely to be correct because when cells are grown on matrices with elastic characteristics similar to those shown above, cells take on or retain differentiated characteristics appropriate for the cell type and matrix [14;16;17].

Current State of Renal Biophysics

Kidneys have a characteristic texture that becomes stiff or hard with progression of disease. In the late 1970's and early 1980's, renal glomerular capillary pressures were found to be increased in renal reduction models [18-20]. Subsequently, assessment of glomerular compliance of glomeruli from remnant kidneys showed increased compliance compared to control glomeruli [21]. In proteinuric disease models (PAN and nephrotoxic serum nephritis) and in response to agents that were either toxic to cells or that would cause proteinuria, glomerular capillaries became fragile, rupturing with minimal stretch [22].

Recently, studies using atomic force microscopy (AFM) and related methods demonstrated reduced stiffness of conditionally immortalized glomerular podocytes from a mouse model of HIV-associated nephropathy (Tg26) and podocytes from *Actn4*^{-/-} mice [23], (Unpub. Obs, M.R. Pollak, R.T. Miller, and P.A. Janmey). At a stage of the disease that showed no detectable pathological changes, glomeruli from Tg26 mice, *Col4a3*^{-/-} mice, *Col XVIII*^{-/-} mice, and protamine-treated glomeruli were 20 - 30 % softer than normal glomeruli (1.5 – 2.0 kPa compared with 2.5 kPa, as measured by AFM and micro-indentation)[24;25] and (Unpub. Obs, P.A. Janmey and R.T. Miller). The relative contributions of glomerular cells and matrix (glomerular basement membrane) to the overall elastic characteristics of glomeruli is an area of active investigation.

These results suggest that increased mechanical deformability of podocytes and glomeruli could be a common feature of a number of renal diseases that would make these structures more susceptible to hemodynamic injury. It could also represent a mechanical environment that is inhospitable to normal glomerular cells and results in apoptosis or dedifferentiation, or that acts as a stimulus for abnormal matrix production and stiffening. The late stages of injury in human diseases and these models are characterized by fibrosis, loss of glomerular and interstitial capillary and tubule function, nephron loss, and ultimately shrunken, hard remnants of kidneys. An important question is, how does tissue softening, also seen in burns, myocardial infarcts, and trauma, lead to a stiff, fibrotic state?

Essentials of Mechanical Signaling

Sensing the extracellular mechanical environment involves multiple mechanisms and sets of proteins that change conformation in response to deformation. In some cases such as stretch-sensitive ion channels, deformation results in increased activity and Ca conductance. In others such as talin, vinculin, p130CAS, filamin, or fibronectin, stretching or deformation leads to unfolding of the protein and exposure of formerly hidden binding sites that can cause reinforced interactions with existing proteins or interactions with new proteins leading to changes in cell structure [26-29]. In general, increased matrix stiffness leads to increased

assembly and contractility of the actin cytoskeleton, increased tyrosine phosphorylation (particularly p125FAK), and increased activity of Rho and its down-stream targets [30].

Although an over-simplification, cells contact matrix via integrins and other cells via cadherins. Integrins and cadherins are the central proteins in large complexes that communicate mechanical signals to cells, and through which cells alter their adhesion to matrix or other cells. In both cases, integrins and cadherins, a complex of proteins with four basic functions, adhesion to extracellular matrix or other cells (e.g. integrins or cadherins), actin linkage (e.g. talin and vinculin), actin polymerization (e.g. mDia1 or Arp2/3), and signaling (e.g. P125FAK, Rho, or Rac) are required for sensing and responding to mechanical signals that lead to altered physical characteristics of cells (E_{Mod}) and other responses (eg motility, spreading, growth, and division) [27;31]. Four integrin-based structures are described, nascent adhesions, focal complexes, focal adhesions, and fibrillar adhesions (Figure 2) that represent progression and maturation from small, initial areas of cell-matrix adhesion to large, relatively rigid regions of the cell where the actin stress fibers are fixed to a matrix-bound integrin-based protein complex. Mechanical force or tension, generated by actin polymerization and non-muscle myosin or external tension in other structures, is essential for development of these structures with increased force leading to increased integrin aggregation, and recruitment of increased amounts of integrin and actin binding proteins (e.g. talin and vinculin), actin polymerizing proteins (e.g. mDia1 and Arp2/3), signaling proteins (e.g. FAK and Src family kinases) [32].

Long-term responses to mechanical signals leading to gene regulation rely on multiple signaling proteins that also function in other pathways [12;16;30;30;33]. Stretch-sensitive Ca channels affect intracellular Ca levels (Ca_i), and a number of pathways that regulate transcription including those involving CaMKII, PKC, and calmodulin are Ca-sensitive. Integrin and cadherin-dependent signaling activates pathways involving non-receptor tyrosine kinases and Rho and ROCK that can affect transcription via SRF (Serum Response Factor) that binds to the serum response element (SRE) [30;34]. Mechanical stress can be transmitted directly from structures at the cell membrane (focal adhesions, points of cell-cell contact) to the nucleus by cytoskeletal elements that connect to the nuclear lamin network via the LINC (linker of the nucleoskeleton and cytoskeleton) complex.

The activity of two transcription systems, MAL/MRTF (Myocardin and Myocardin-Related Transcription Factors A and B a.k.a. megakaryocyte leukemia protein, MAL), and YAP/TAZ (Yes-Associated Protein and its homolog TAZ, Transcriptional co-Activator with PDZ binding motif) are controlled in part by the state of the cytoskeleton. The myocardin/MRTF/MAL proteins are transcriptional coactivators that bind SRF, and are held in the cytoplasm by G-actin. In response to mechanical force and Rho signaling, actin polymerization and contraction deplete the pool of G-actin and, the MRTF proteins move to the nucleus where with SRF they activate genes involved in hypertrophy and fibrosis such as α -SMA (α -smooth muscle actin) [30]. YAP/TAZ are part of the Hippo pathway, a kinase cascade, that stimulates cell proliferation, inhibits apoptosis, mediates contact inhibition, and is involved in development and determining organ size [35;36]. Although regulated by G protein-coupled signaling systems, cadherins, and other pathways, these two proteins also respond to the mechanical state of cells by localizing either to the cytoplasm of

nucleus[36-39]. In soft cells, cells on soft matrix, or cells restricted from spreading, YAP and TAZ are restricted to the cytoplasm. In stiff cells, cells on a stiff matrix, or spread cells, they migrate to the nucleus where they activate genes involved in proliferation and fibrosis such as CTGF [39]. YAP and TAZ are important in developmental pathways where they control expression of gene involved in early nephrogenesis [40;41]. YAP is expressed in podocytes in intact glomeruli as well as conditionally immortalized podocytes in culture, and in both cases has a nuclear localization. Nuclear localization is associated with cell survival, while loss of nuclear localization and cytoplasmic localization is associated with apoptosis [42;43] How YAP/TAZ cellular localization relates to mechanical stimuli is not known.

Interactions of Matrix Chemistry and Mechanics

Matrix provides both chemical (growth factors, matrix components) and mechanical (E_{Mod}) signals. The E_{Mod} of the matrix is determined by the nature of its components (different collagens, elastin, proteoglycans), the amount of these components, and the degree to which they are cross-linked. As a collagen matrix stiffens, cells spread and stiffen to a limit determined by the cell type (eg fat cells are always softer than muscle cells) and the stiffness of the matrix. On collagen, the E_{Mod} of a cell does not exceed the E_{Mod} of the matrix (Figure 3). For example, if a muscle cell that can have an E_{Mod} of 10 kPa on plastic is grown on a matrix that is 5 kPa, the E_{Mod} of the muscle cell will be approximately 5 kPa [33]. On matrices with different chemical composition, e.g. collagen vs. fibronectin, the rate of cell stiffening as a function of matrix stiffness is markedly greater on collagen than on fibronectin, although the cell spread areas are similar [33]. This differential response to matrix components has cellular physico-chemical correlates[28]. For many cell types when bound to their substrate only by integrins, the stiffness of a cell does not exceed that of its matrix. However, the combination of fibronectin and hyaluronic acid allows cardiac myocytes, fibroblasts, and endothelial cells on 0.3 kPa gels to stiffen to several kPa. The cells have appearances similar to cells grown on glass that are spread broadly and have extensive stress fibers, but unlike cells on a stiff collagen or fibronectin matrix, YAP remains cytoplasmic, a characteristic of soft cells, or cells on a soft matrix (Figure 3) [5].

The effect of elasticity in tissue function

Myofibroblasts

An increase in matrix, primarily fibrillar collagen, is a part of wound healing, serving to prevent tissues from disassembly during injury, or repair tissue disruption [7;44-46]. Fibrillar collagen and other matrix components come primarily from myofibroblasts, cells that are not present in normal tissues. Cells such as pericytes, resident fibroblasts, and hepatic stellate cells respond to injury, become activated, and develop into myofibroblasts. Myofibroblasts are characterized by expression of α -smooth muscle actin (α -SMA), types I and II collagen, proteoglycans, ED-fibronectin, and a number of cytokines, have prominent stress fibers that contain α -SMA, mature fibrous focal adhesions, high contractility, and reduced levels of COX-2[3]. In most studies, a matrix E_{Mod} of 5 - 10 kPa is required for development of β -actin fibers (protomyofibroblasts), although this value may vary depending on the tissue [7;47]. At that point, the presence of TGF- β with continued tension

leads to mature myofibroblasts. Importantly, TGF- β alone is not sufficient to induce development of myofibroblasts, but requires a stiff matrix. TGF- β is bound by matrix (fibrillin, proteoglycans, and fibronectin) and specific TGF- β -binding proteins (Latency Associated Protein, LAP, which binds integrins $\alpha_v\beta_5$ and $\alpha_v\beta_6$). Release of TGF- β by LAP depends on the elasticity of the matrix and the contractive force of the cells [47]. On a stiff matrix ($> \sim 10$ kPa), the cell (with integrins) contracts against the matrix and deforms LAP releasing active TGF- β .

Normally once healing is complete, activation of myofibroblasts and their numbers decrease, and production of matrix and wound evolution become stable so that tissue function is essentially normal. Turn-off mechanisms may include expression of DKK-1, a ligand for LRP-5 and 6, co-receptors that inhibit the Wnt- β -catenin pathway, that blocks myofibroblast activation [48]. Alternatively, fibrosis can progress resulting in loss of tissue or organ function via the stiff matrix- LAP-contractile cell-TGF- β release positive feedback loop mechanism. Why this occurs in some cases is not understood, but may relate to persistent irritation or injury with prolonged presence of inflammatory cells or cytokines. A component of the problem may also be that the matrix becomes resistant to remodeling (proteolysis) as a result of increased cross-linking and stiffness [44]. In one report valvular myofibroblasts became dormant when they were transferred from 32 kPa matrix to a 7 kPa matrix, so that reduced stiffness of the matrix may be sufficient to arrest fibrosis [49].

Liver Disease

The elastic modulus of normal liver is 0.4 – 0.6 kPa, but following injury and fibrosis can increase to as much as 15 kPa[9;50-52]. The normal elastic environment of the liver, 0.4 - 0.6 kPa is not sufficient to induce differentiation of stellate cells and portal fibroblasts into myofibroblasts [1;7;45]. In CCl₄ injury a model for liver fibrosis, liver tissue becomes significantly stiffer before collagen expression increases. Part of the early increase in stiffness was prevented by inhibitors of lysyl oxidase, an enzyme that cross-links collagen and elastin [9]. Increasing matrix E_{Mod} to 2.5 kPa induced differentiation of hepatic stellate cells to myofibroblasts [1]. Induction of myofibroblasts was independent of matrix chemistry (Col I, fibronectin) and presence of TGF- β , although TGF- β increased the responses to matrix stiffness[1]. Hepatic fibrosis can regress when the initial injurious agent is eliminated. In the CCl₄ model, approximately half of the myofibroblasts undergo apoptosis, while the remainder regress, and become quiescent through up-regulation of anti-apoptotic genes, although the cells do not fully reach their pre-activated state[53].

The expression of lysyl oxidase increases early in liver injury, in some studies by 1 day post injury[54;55]. The degree of matrix crosslinking can have marked effects on matrix mechanics and cell behavior in the absence of new matrix synthesis[13]. Increased activity of lysyl oxidase and other enzymes with similar functions may provide the first step in myofibroblast activation and repair, stiffening of existing matrix, allowing the subsequent full development of myofibroblasts. The primary sources of lysyl oxidase in the liver appear to be resident or activated stellate cells and portal fibroblasts[55].

Wounds

Although in late stages of wound repair and pathologic fibrosis wounds may have an E_{Mod} of 1 – 20 kPa or more, early wounds are soft [2]. Studies of surgical wounds in piglet skin show that at 5 days, the granulation tissue between sutures where there is more tension is ~ 0.45 kPa, while at suture sites where the tension is minimized, the E_{Mod} of the granulation tissue is initially ~ 0.3 , and increase to ~ 1 kPa at 14 days [56]. The value of 1 kPa for granulation tissue has been cited in other sources[7], and is likely to be accurate because of the mechanical instability of injured tissue such as seen with rupture of left ventricular walls following transmural myocardial infarcts, conversion of bland to hemorrhagic strokes, and the gelatinous nature of acutely rejected kidneys.

Summary

In disease, the elastic characteristics of tissues including glomeruli are altered at an early time point. The mechanical environment is important for compensation for, and resolution of injuries. Softening of glomeruli early in disease may be equivalent to wounding of other tissues in that glomeruli have specific requirements for mechanical integrity that must be maintained. Strategies for repair in all tissues as are likely to be similar and involve myofibroblast development permitted by a stiff environment with cytokines such as TGF- β . Increased lysyl oxidase activity and expression with increased collagen crosslinking and increased collagen production are essential components of the stiff environment required for myofibroblast development as demonstrated in models of cirrhosis [1;9;57]. Although work is under way in glomeruli, inhibition of fibrosis with lysyl oxidase inhibitors has been demonstrated in models of cirrhosis and vascular disease [9;57]. Similarly, understanding the normal processes by which matrix is resorbed from wounds may be valuable. Finally, combinations of matrix components such as fibronectin and hyaluronic acid that override signals from a soft matrix to create a “stiff cell” mechanical environment may also contribute to responses to injury, and could also be therapeutic targets [5;58;59].

Acknowledgments

The authors thank Scott M. Grundy for helpful comments and discussion.

Financial Support and Sponsorship. This work was supported by RO1 DK083592 (RTM and PAJ), R01 EB017753 (PAJ), the Veterans Affairs Administration, and the Charles and Jane Pak Center for Mineral Metabolism and Clinical Research.

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Key Points

The elastic environment of cells is an essential factor that with the biochemical environment determines cell behavior (growth, differentiation, survival).

Each tissue has characteristic elastic properties that presumably serve its function.

Fibrosis or scarring are the end point of various forms of injury or disease, and are associated with myofibroblast activation, a process that requires a stiff matrix.

Diseased glomeruli or tissue wounds at early time points, are characterized by increased softening of tissue, yet progress to fibrosis with increased stiffness at late time points.

How injured tissue progresses from soft to recover their mechanical integrity or progress to scarred and fibrotic is not understood, but the process is more complex than simple myofibroblast activation, and may require early modification of the injured tissue for mechanical integrity and to support repair by myofibroblasts and other similar cells.

Figure 1. Comparison of the elastic moduli (E_{Mod}) of several normal and diseased tissues

The elastic moduli of a number of normal adult tissues is compared to diseased tissues using a log scale of elastic modulus (E_{Mod}) that ranges from 0 to 10^9 Pa. Normal tissues are shown in black above the scale, and diseased tissues are shown in blue below the scale. The values were obtained using rheometry, atomic force microscopy, or micro-indentation.

Figure 2. Essentials of mechanical signaling

This figure shows the basic molecular machinery that senses and responds to matrix-generated mechanical signals. When a cell encounters a matrix, integrins bind proteins in the matrix, and additional proteins on the cytoplasmic surface aggregate forming a focal complex (Top). A focal complex contains integrins that connect the extracellular matrix to actin fibers as well as additional proteins that lead to activation and aggregation of integrins and that link them to actin fibers. These proteins include talin, vinculin, and paxillin. Kinases and phosphatases, also important for these processes, are not shown for simplicity. In the presence of force, probably generated by actin polymerization, additional integrins aggregate and bind to F-actin fibers and non-muscle myosins leading to formation of a focal adhesion (Middle). The cell surveys its mechanical environment with periodic contraction of actin and non-muscle myosin stress fibers, which are attached to the integrins that pull against the matrix. To sense force, the complexes must be separated by > 2 μm . Over time, and as a result of mechanical force arising from the actin fibers and non-smooth muscle myosins, the aggregation of these additional proteins results in development of mature focal

adhesions (Bottom). Additionally, proteins, such as α -actinin, filamin, and cortactin, cross-link actin fibers thereby adding mechanical strength to the actin cytoskeleton and consequently the cell overall. Filamin and α -actinin also participate in linking actin fibers to integrin β subunits.

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Figure 3. Responses of normal cells to matrix elasticity

The top two images show the responses of normal cells to soft and stiff matrices. An abnormal cell (bottom image) that is unable to sense matrix stiffness. In the top image, a cell is shown on a soft matrix, which is represented by a wavy black line, indicating that the cell can contract against the matrix and deform it. This cell has only few focal adhesions (red squares) and actin fibers (green arrows). When the same cell is placed on a stiffer matrix, which the contractile apparatus of the cell cannot deform (second image), the number of focal adhesions increases. The number of actin-non-muscle myosin stress fibers and their thickness also increases, leading to cell spreading and stiffening. In disease states such as cancer and scarring, cells might encounter abnormally stiff matrix and therefore take on abnormal mechanical and cell biologic characteristics. The third image shows a cell that cannot sense or respond to matrix-generated mechanical signals on a stiff matrix. The cell remains soft with only a few focal adhesions and actin fibers. Cells with these characteristics are for example found in filamin-null M2 melanoma cells, cells that lack integrins, in glomerular podocytes from a mouse model of HIV-associated nephropathy, α -actinin-4-null mice, and in cells without functional non-muscle myosins. These cells all have defects in

adhesion, migration, and show increased susceptibility to injury by mechanical force. The bottom image shows a normal cell on a soft matrix that is composed of fibronectin (FN) and hyaluronic acid (HA). In this case, the matrix induces the cell to develop focal adhesions and stress fibers and become stiffer than its matrix. This situation could arise in a wound where FN and HA are present. In cells on a stiff matrix (stiff cells) YAP is localized to the nucleus, In all other conditions, soft matrix, soft cells, or stiff cells on a soft matrix composed of fibronectin and hyaluronic acid, YAP is cytoplasmic.