CHAPTER 7

Functional Interactions of the Extracellular Matrix with Mechanosensitive Channels

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I. OVERVIEW

Mechanical stimuli generate responses in many types of mammalian cells that interact with the extracellular matrix. As the extracellular matrix is a potential force conductor, in many tissues mechanical loads can be directed through fibrillar matrix proteins to matrix receptors such as integrins. These transmembrane receptors direct forces into the cytoskeleton and can influence the gating properties of mechanosensitive channels in the adjacent plasma membrane. Stretch-sensitive channels colocalize with integrins and

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functional studies indicate that stable matrix-integrin attachments are required for stretch activation. We review here the mechanical properties of prominent matrix proteins, the nature of cell attachments to the matrix, how cell-matrix attachments interact with the cytoskeleton to regulate stretch sensitivity of mechanosensitive channels and contemplate how matrix proteins may interact with mechanosensors to effect mechanotransduction. These findings point to conserved regulatory mechanisms by which cells in vertebrates respond to external forces and convert these forces into signals that mediate alterations in the structure and function of connective tissues.

II. MECHANOTRANSDUCTION

Cellular mechanics can be considered as a three-step process comprising mechanosensation, mechanotransduction, and mechanoresponse (Vogel and Sheetz, 2006). Mechanosensation involves the ability of cells to detect forces and to explore the topography of the extracellular matrix. This information about the physical environment is then translated into intracellular biochemical signals, a process known as mechanotransduction that leads to the generation of cellular responses (mechanoresponses) which may impact the structure and function of the extracellular matrix itself. Target cell adaptation and desensitization of mechanosensitive channels to constant or repeated mechanical stimulation in connective tissue fibroblasts has been reported (Glogauer *et al.*, 1997) and may prevent "information overload" including overt Ca^{2+} induced cell death. Consequently, cellular adaptations to applied mechanical forces, including the regulation of mechanosensitive channel function, is important for optimizing cellular responses to fluctuations in the physical environment of cells (Alberts *et al.*, 2002).

Mechanosensing, mechanotransduction, and mechanoresponses comprise tightly integrated processes involving extracellular matrix, cytoskeletal, and signaling proteins that ultimately provide cells with the ability to respond and adapt to applied physical forces. Direct mechanical influences that evoke cellular responses may impact the cell membrane and include stress, strain, fluid shear, hydrostatic pressure, volume change, osmolarity, and geometry sensing (Orr *et al.*, 2006). In particular, mechanoresponses are dependent on the magnitude and rate of loading or complexity of surface geometry as well as the age and differentiation state of the cell (Clements *et al.*, 2001). Some of the earliest mechanoresponses following mechanical stimulation of connective tissue cells are transient increases in intracellular calcium or reorganization of the cortical cytoskeleton. Longer term responses include the secretion of cell mediators into the matrix such as prostaglandin E2 and interleukin-1, signals that may increase cell proliferation and upregulate

the synthesis of matrix components, matrix degrading enzymes, and growth factors (Chiquet, 1999). Mechanoresponses are complex and diverse but the pathways can be broadly characterized as associated with physical force or those involved in geometry sensing and cell motility (Vogel and Sheetz, 2006).

Cytoprotection is a group of individual mechanoresponses that serve to protect the cell from potentially harmful mechanical forces, possibly leading to cell death and loss of tissue homeostasis. These responses can include reinforcement of the cell membrane to prevent excessive mechanical deformation (e.g., assembly of actin filaments in orthogonal arrays; Glogauer *et al.*, 1998) or the operation of stretch-inactivated ion channels (Gu *et al.*, 2001). Some of these adaptations include the ability of cells to repair damaged cell membranes and to survive high amplitude forces without undergoing cell death (Kainulainen *et al.*, 2002; McNeil and Kirchhausen, 2005) and the ability to remodel the extracellular matrix in response to applied forces (Ozaki *et al.*, 2005). Cytoprotection manifests in the response to sustained loading which in fibroblasts results in an upregulation in the expression of actin cross-linking proteins that enhance local membrane rigidity via subcortical actin filaments (D'Addario *et al.*, 2001).

It seems likely that at least some of the proteins involved in mechanotransduction can form aggregates that optimize the transmission and processing of mechanically induced signals (Tzima *et al.*, 2005). Mechanosensitive channels are functionally linked to transmembrane proteins that provide anchorage for cells to the extracellular matrix (e.g., integrins; Glogauer *et al.*, 1997) or for intercellular adhesions (e.g., cadherins; Ko *et al.*, 2001). These attachment proteins are linked by actin-binding proteins to the subcortical actin network and form complexes with proteins that regulate tension in the cell membrane and the conductance of mechanosensitive channels. Indeed, one of the central notions of the regulation of ion channel conductance by attachment proteins and the cytoskeleton is that cell membranes are important targets for delivery of exogenous mechanical loads (Martinac, 2004) that can then be translated into membrane tension. Conceivably, increased membrane tension can lead to increased ion channel conductance, and ultimately, altered gene expression.

In cells of connective tissues, force transmission through fibrillar extracellular matrix proteins (Provenzano and Vanderby, 2006) can be focused on to discrete patches of the cell membrane (Fig. 1) through the organization of matrix receptors into clusters such as focal complexes or the more mature and extensively cross-linked focal adhesions (Galbraith *et al.*, 2002). As the formation of focal adhesions is dependent on cell-generated tension by myosin motors bound to actin, why do cells anchored to the matrix not spontaneously activate mechanosensitive channels as has been reported for

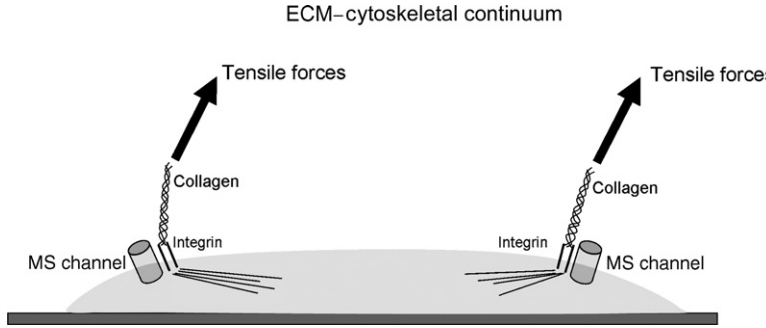

FIGURE 1 Diagram to illustrate potential functional relationship between tensile forces applied through collagen fibrils to integrins and how these tensile forces may impact adjacent mechanosensitive ion channels.

migrating cells (Lee *et al.*, 1999)? Presumably, if the matrix to which cells are anchored is sufficiently stiff, cells resist the deformations generated by internal forces (Pelham and Wang, 1997) and the stretch channels will not be activated. Evidently, there are very precise balances in cells between matrix-applied forces, cell deformation, membrane tension, and cytoskeletal elements that resist deformation (Coughlin *et al.*, 2006). The impact of these balances on alterations of cell metabolism likely depends on the activity of the cell at any given moment (migrating, quiescent, dividing, undergoing differentiation, subjected to tension), its relationship with adjacent cells (via intercellular adhesions), and its attachment to the substrate (extracellular matrix). We explore below how elements of the extracellular matrix not only provide force transmission to cells but also aid in force sensing and in determining cellular responses to force. The generation of mechanically induced responses may facilitate the migration of cells through the matrix and the organization of tissues, particularly as regulated by matrix adhesion receptors that are deeply involved in mechanotransduction processes (Katsum *et al.*, 2004).

III. MECHANOSENSITIVE CHANNELS IN CONNECTIVE TISSUE CELLS

Mechanical loading of bone leads to tissue remodeling (Lanyon, 1984) as does force application to soft connective tissues such as the periodontal ligament (Bumann *et al.*, 1997). Connective tissues are evidently impacted by applied forces and are an attractive system for determining how force leads to alteration of gene expression and matrix remodeling. Bone remodeling in particular has highlighted the characterization of mechanosensitive

ion channels in cultured osteoblastic cells and osteoclasts. By patch clamping, Davidson *et al.* (1990) identified three classes of channels in osteoblastic cells that were characterized on the basis of conductance, ionic selectivity, and sensitivity to membrane tension. The coexistence of mechanosensitive nonselective cation and K^+-selective channels in these cells indicated that applied forces could promote either membrane hyperpolarization, depolarization or a multiphasic response, depending on the density of the channels in the deformed region of the membrane. One of the difficulties with these types of patch-clamp studies is to relate the magnitude of the membrane deformations to physiological bone matrix turnover since the stresses induced by the patch pipette might be very different than those encountered *in vivo* (Sachs, 1988). Ypey *et al.* (1992) also found stretch-activated K^+-selective channels in chick osteoclasts, thereby providing the basis for a cellular system by which both synthetic and resorptive cells of the bone matrix could be integrated into a stretch-sensitive tissue remodeling system.

In studies that were related to physiological regulatory systems of osteoblasts, Duncan and colleagues (Ryder and Duncan, 2001) used shear forces (1 dyne/cm^2) applied to cultured murine osteoblastic cells to examine interactions between parathyroid hormone and fluid shear-induced Ca^{2+} signals. Notably, parathyroid hormone is an osteotropic hormone that, when combined with mechanical stimulation, increases bone mass. Their data indicated that parathyroid hormone enhanced the Ca^{2+} response to shear force by protein kinase C modulation of mechanosensitive cation channels and voltage-sensitive Ca^{2+} channels. While the identity of these channels has not been determined, reconstitution experiments using expression of the α-subunit of the epithelial Na^+ channel from osteoblasts demonstrated a Ca^{2+}-permeable, cation-selective, stretch-sensitive channels with some of the expected properties of mechanosensitive channel in intact osteoblasts. RT-PCR, Western blotting, and immunohistochemistry have been used to confirm that human-derived osteoblasts and MG63 cells express TREK-1 mRNA and protein. In human osteoblasts, functional expression of TREK-1 indicates that these channels may contribute to the resting membrane potential of human osteoblast cells (Hughes *et al.*, 2006). Recent data indicate that the focal adhesion kinase pp125FAK interacts with the large conductance Ca^{2+}-activated hSlo K^+ channel in human osteoblasts, thereby suggesting a potential role in mechanotransduction (Rezzonico *et al.*, 2003). Evidently, there is an important role for mechanosensitive channels in the physiological regulation of connective tissues but currently, the identity and molecular regulation of these channels, as well as their functional interactions with matrix elements are not well defined. Below, we consider the molecular determinants of connective tissues that may be important for the regulation and function of mechanosensitive channels.

IV. THE EXTRACELLULAR ENVIRONMENT OF CELLS

Cells of soft and mineralized connective tissues reside within an extracellular matrix that they may elaborate themselves (e.g., dermal fibroblasts in the lamina propria of the skin or osteoblasts in the bone matrix) or into which they have migrated (e.g., the myofibroblasts in the provisional matrix of healing wounds) or in which they become embedded during tissue formation (e.g., chondrocytes of cartilage; osteocytes of mineralized bone). In other types of tissues, epithelial cells (e.g., gastrointestinal-lining cells) and endothelial cells (blood vessel-lining cells) attach to underlying connective tissue matrices that provide anchorage, metabolic exchange, and the molecular factors required for growth and differentiation. Thus, in many tissues that are subjected to either gravitational, muscle, or endogenous cell-generated forces, the extracellular matrix, in addition to its roles in anchorage, metabolism, and chemical signaling, also provides a medium through which mechanical signals can be directed to impact cell function. Notably, many types of connective tissue cells such as fibroblasts exhibit extensive intercellular junctions (Beertsen and Everts, 1980; El-Sayegh *et al.*, 2005), enabling them to function as part of a syncitium and thereby facilitate transmission of mechanical forces between cells and throughout a tissue (Xia and Ferrier, 1992; Boitano *et al.*, 1994). Consequently, in connective tissues, mechanosensing and mechanotransduction processes may involve force transmission through fibrillar elements of the extracellular matrix and/or by direct force transmission through intercellular adhesions.

Sensing mechanical signals from the immediate matrix environment is important not only for appropriate cellular adaptation and mechanoprotective phenomena (McNeil and Kirchhausen, 2005) but for linkage of mechanotransduction processes to degradation (e.g., phagocytosis; Beningo and Wang, 2002) and tissue synthesis and remodeling (Guo *et al.*, 2006). A combination of synthetic and degradative processes is involved in extracellular matrix remodeling and for mediating adaptations of the biomechanical properties of the matrix in response to metabolic and functional demands (Goodship *et al.*, 1979). Collectively, these adaptive processes act to protect the tissue as a whole, particularly if subjected to high amplitude mechanical loading. This is an important determinant of tissue homeostasis since during physiological ageing there is an overall decrease in cell density and global cellular responsiveness to external stimuli. Accordingly, tissue repair responses may be outpaced by accumulating fatigue failure in fibrillar matrix proteins, thereby illustrating the vital role of cells in maintaining the biomechanical health of tissues in both developing and aging mammals (Clements *et al.*, 2001, 2004).

Mechanical forces that act on cells vary in frequency, amplitude, and duration and are in turn further diversified by the composition and biomechanical

properties of the extracellular matrices that "deliver" forces to resident cells. Connective tissues exhibit, in general, three types of matrices: (1) mainly fibrillar (e.g., tendons and ligaments); (2) a mixture of fibers and specialized ground substance (e.g., bone); and (3) fluids (e.g., blood) (Nordin and Frankel, 1980). The relative abundance of fibers and, in particular, the mineralized components of connective tissues have very large effects on the mechanical properties of specific connective tissues and on the delivery of mechanical forces to cells. While shear forces in the arterial blood stream may be transmitted directly to circulating blood cells, compressive, bending, torsion, and tensile forces are largely dissipated after delivery to osteocytes embedded in the bone matrix. Indeed, the ability of osteocytes to function as tissue mechanosensors for bone relies in part on their exquisite sensitivity to minute changes in fluid flow through the surrounding osteocyte lacuna (Han *et al.*, 2004). In health, there is an optimal distribution of forces between matrix and cell. The extracellular matrix provides stress shielding to cells but, in situations of high amplitude mechanical force loading, forces can exceed cell- and matrix-dependent protective systems to induced cell loss by apoptosis (Chen *et al.*, 2003; Goga *et al.*, 2006).

Extracellular matrix composition is governed by functional demands which in turn determine the physical nature of the matrix. Reciprocal relationships between function and form, and the physical properties of connective tissues, impact not only cells that reside within connective tissues such as fibroblasts and osteocytes, but also regulate the forces applied to cells which are attached to connective tissues such as endothelial or epithelial lining cells. Bone, cartilage, enamel, and dentine are subjected to very high compressive forces and deformation of these tissues is resisted by their high content of hydroxyapatite, calcium phosphate lattices that are extremely rigid. Many load-bearing tissues also have a high concentration of collagen (Oloyede and Broom, 1996). For fibrillar collagens, the tight, triple helical arrangement of tropocollagen endows collagen fibers with an extraordinarily high tensile strength and resistance to shear deformation (Li *et al.*, 2005). Collagens also exhibit critical molecular domains that are required for cell attachment, differentiation and have been shown to associate with mechanosensitive channels (Liu *et al.*, 1996). Collagen fibers are, however, very narrow and are subject to buckling under compression; when subjected to excessive mechanical loading, collagen fibers exhibit thickening and deformation (Kaab *et al.*, 2000). In cartilage, tissue resistance to compressive deformation is dependent on the function of a gel phase comprising proteoglycans whose long and highly charged side chains can bind high molar concentrations of water. The combination of collagen fibrils and the gel matrix in articular collagen and intervertebral discs provides these tissues with enhanced viscoelastic properties, which are seen as creep and stress relaxation during

complete recovery from deforming loads (Adams *et al.*, 1996; Kerin *et al.*, 1998).

In addition to the extracellular matrix that surrounds connective tissue cells, many cell types exhibit a surface coating of matrix molecules such as glycoproteins (e.g., fibronectin), proteoglycans (e.g., heparan sulfate), glycolipids, and other cell adhesive molecules that comprise the glycocalyx, a structure that varies in thickness in different cell types and is modulated by the level of cell differentiation (Sengupta *et al.*, 2000) (Fig. 2). In endothelium, shear forces from blood flow are dampened by the glycocalyx before they are delivered to the cell membrane, underlining the importance of the glycocalyx in regulating mechanical signaling. The glycocalyx is composed of proteins with highly glycosylated extracellular domains, and some proteins with membrane-spanning domains and short cytoplasmic tails. Notably, in endothelial cells, heparan sulfate proteoglycan may play a critical role as a mechanosensor in mechanical signaling events related to fluid flow (Florian *et al.*, 2003). The glycocalyx overlying the endothelial surface layer can extend up to several microns peripheral to the plasma membrane; when the glycocalyx is intact, it can dissipate fluid shear at the plasma membrane of endothelial cells to near zero levels (Tarbell and Pahakis, 2006). The total bulk of charged carbohydrate moieties offers resistance to cellular deformation, while specific end sugars may serve as mechanosensors. For cells to detect forces within the extracellular matrix, it would seem that mechanosensors must be located at the glycocalyx surface or protrude beyond.

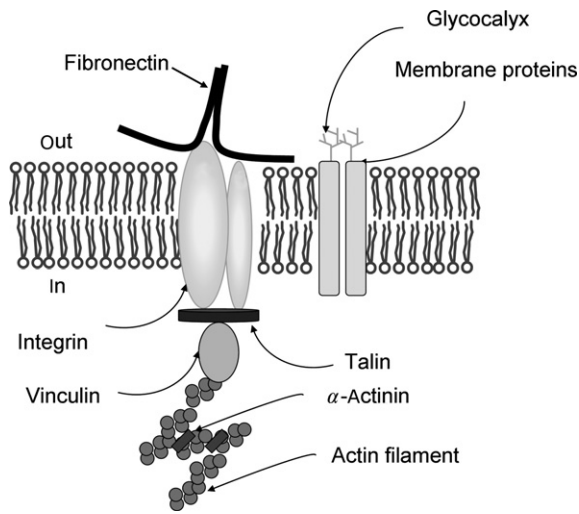

FIGURE 2 Diagram to show cell surface glycocalyx and possible interactions with membrane-associated cell adhesion receptors.

V. FORCE TRANSMISSION FROM MATRIX TO CYTOSKELETON

An effective transducer of mechanical forces that can ultimately alter cellular metabolism would be expected to exhibit membrane-spanning domains and a functional association with the cytoskeleton (Watson, 1991). The integrin superfamily of matrix adhesion receptors seems well-suited for this purpose and these molecules have been considered as critical elements of the mechanosensory machinery (Katsum *et al.*, 2004). Integrins provide a critical link for transmembrane communication between the extracellular matrix and subcortical actin filaments via specialized adhesions that comprise a large array of actin-binding proteins, signaling proteins, and the termini of actin filaments (Galbraith *et al.*, 2002). Integrins and other cell surface matrix receptors can provide a continuum from the extracellular matrix to the actin cytoskeleton (and other cytoskeletal systems) that link the cell exterior to the cell interior as a mechanically coherent unit.

Integrins are heterodimers formed from at least 18 distinct α-subunits and 8 distinct β-units which dimerize in various combinations (Miranti and Brugge, 2002). Integrins are involved in intercellular signaling and exhibit cross talk with other receptors; they also play critical roles in cell survival and regulation of cell proliferation (Miranti and Brugge, 2002). Each integrin exhibits a measure of specificity for binding extracellular matrix ligands. For example, $\alpha1\beta1$, $\alpha2\beta1$, $\alpha3\beta1$, $\alpha10\beta1$, and $\alpha11\beta1$ integrins bind type 1 collagen; some of these receptors play a role in regulation of phagocytosis by fibroblasts (Lee *et al.*, 1996) and in the transmission of mechanical signals from collagen to cells. Fibronectin is a ubiquitous extracellular matrix protein, which is secreted as subunits and assembled extracellularly into a fibrillar network. This assembly process is integrin dependent and is particularly important for assembly of the glycocalyx. Notably, $\alpha v\beta1$ and $\alpha5\beta1$ integrins bind to fibronectin (Mao and Schwarzbauer, 2005). Integrins have a single membrane-spanning domain and short cytoplasmic tails which interact with the cytoskeleton via the actin-binding protein talin. It is not known if all integrins linking the extracellular matrix to the actin cytoskeleton participate in mechanotransduction nor have direct integrin-mechanosensitive channel interactions been identified to date. However, $\beta1$ integrins have been colocalized with epithelial Na^+ channels and voltage-activated Ca^{2+} channels in putative mechanoreceptor complexes in mouse chondrocytes (Shakibaei and Mobasheri, 2003), suggesting the possibility that mechanical interactions between these two groups of molecules may be involved in mechanosensitivity.

A. Focal Adhesions

Studies of extracellular matrix ligand binding in cultured connective tissue cells have shown that integrin clustering acts as a nucleation site, recruiting to the adhesion complexes a large array of signaling molecules such as the

focal adhesion kinase, Src kinases, as well as phosphatases such as SHP-2 (MacGillivray *et al.*, 2000; Herrera Abreu *et al.*, 2006). The nascent focal complexes exhibit assembly of actin filaments and the recruitment of a large array of actin-binding proteins such as vinculin, talin, paxillin, and α-actinin (Zamir and Geiger, 2001). As the adhesions mature into focal adhesions and then super-mature focal adhesions (Dugina *et al.*, 2001), more complex aggregates of proteins including α-actinin are assembled into these tightly adherent protein arrays.

On the basis of matrix adhesion receptors such as integrins, specialized adhesive plaques, like focal adhesions *in vitro* and the fibronexus complex *in vivo*, permit cells to interact with a broad array of extracellular matrix molecules such as laminin, vibronectin, fibronectin, and collagen. When immunostained *in vitro* for vinculin or paxillin, focal complexes appear as tiny spear tips that are distributed on the ventral surface of cells adhering to the underlying substrate. The use of green fluorescent protein tagging has enabled an improved understanding of the structural and temporal aspects of the different molecular components of focal adhesions (Wehrle-Haller and Imhof, 2002), including microstructural analyses and the development of a more sophisticated classification of focal adhesions. This is relevant to the function of mechanosensitive channels in connective tissues since focal adhesions are enriched with signaling molecules and they provide critical force transfer sites into the cellular interior. Further, focal adhesions appear to be crucial for our understanding of cell-mediated matrix remodeling. Notably, actin stress fibers insert into focal adhesions and these organelles enhance the development of tension that is involved in matrix contraction. In healing wounds and morphogenesis, contractile forces are crucial for remodeling of the nascent extracellular matrix and for the generation of strong propulsive forces which are reduced as the adhesions grown in size (Beningo *et al.*, 2001). The converse seems to be the case for focal adhesions involved in contraction of the matrix. In myofibroblasts, they develop into "supermature" focal adhesions, which are considerably larger and are able to exert two- to fourfold greater forces on the extracellular matrix than earlier stage focal adhesions (Hinz, 2006).

B. Selectins

Circulating leukocytes marginate to walls of the blood vessels at sites of inflammation. In order to gain access to the inflamed site, leukocytes adhere to specific regions of the endothelial cell wall and then migrate through intercellular contacts, a process known as extravasation. Leukocytes roll along the endothelial wall and can form more permanent tethering types of

adhesions to the endothelium which is the first stage in their eventual migration across the blood vessel wall and into connective tissues. The $\alpha 4\beta 1$ integrin binds both to fibronectin and to vascular cell adhesion molecule-1 expressed on endothelial cell surfaces. The integrin-vascular cell adhesion molecule bond strength is of the order of >50 pN and thus provides a firm attachment for cells in spite of shear forces from flowing blood (Zhang *et al.*, 2004).

Other binding ligands for endothelial cell adhesion molecules are transmembrane glycoproteins known as selectins that are also expressed on the cell surface of leukocytes. In order to promote leukocyte adherence, selectins require exposure to a threshold level of shear force. Slip bonds are weak noncovalent bonds that break under low shear stress, but allow cells to adhere briefly, "survey" the local endothelium, and then roll on. Catch bonds are much stronger adhesions that form under high shear force and are responsible for tethering. Depending on the local shear forces experienced by adherent cells, L-selectins can rapidly switch between expression of slip bonds and catch bonds, and thus may function as highly effective mechanosensors. In addition, they can activate shape change within the cells by modulating the organization of the actin cytoskeleton. This can be mediated directly by the cross-linking actin-binding protein α-actinin, or indirectly through ezrin-radixin-moesin proteins (Ivetic and Ridley, 2004; Yago *et al.*, 2004). This putative role of selectins as mechanosensors is highlighted by studies of adhesion of eosinophils to interleukin 4-stimulated endothelial cells. In this model, adhesion of eosinophils to endothelial cells induced shear-dependent increases of endothelial cell intracellular calcium and increased phosphorylation of extracellular signal-regulated kinase. Further, ligation of either vascular cell adhesion molecule-1 or E-selectin induced a shear-dependent increase in ERK2 phosphorylation in cytokine-stimulated endothelial cells (Cuvelier *et al.*, 2005). Collectively, these data indicate that selectins can regulate the activity of mechanosensitive Ca^{2+}-permeable channels in endothelial cells in a collaborative manner requiring inputs from multiple molecular sources.

VI. EXPERIMENTAL MODELS OF FORCE APPLICATION TO CONNECTIVE TISSUE CELLS

Although a great deal has been learned by examining regulators of mechanical signaling in intact organisms (e.g., stretch activation of transcription factors in *Drosophila*; Somogyi and Rorth, 2004), improved understanding of the mechanisms of cell biomechanics has largely involved the development of *in vitro* model systems. A wide variety of experimental

force application systems have been designed and several complex surfaces have been created to study geometry sensing (Liu *et al.*, 1999; Rovensky *et al.*, 2001). While cells *in vivo* may experience several types of deforming forces simultaneously, several laboratories have designed experimental devices that resolve force application into unidirectional mechanical stretch (static or cyclic) or shear stress. Several systems can effect repetitive as well as static loading and can localize loading to discrete regions of the cell membrane by "prodding" with micropipettes. This technique can be effected, for example, with micropipettes mounted on micromanipulators and can involve simple touching and analysis of cell responses by, for example, microscopic fluorimeter measurements of fluorescent dyes that report $[Ca^{2+}]_i$ (Xia and Ferrier, 1992). These techniques can be used mainly for studies of single cells and as such are largely restricted to measurement of ion fluxes. Biochemical analyses require typically $>5 \times 10^5$ cells per analysis. High-resolution electrophysiological measurements such as patch clamping provided the initial data on mechanosensitive channels in connective tissue cells (Guharay and Sachs, 1984) but as this technique also damages the cell membrane, inferences about cell-matrix interactions are not feasible (Sokabe *et al.*, 1991).

The most commonly employed method for studies of mechanical signaling in connective tissue cells is the use of devices that subject cells to substrate elongation (MacKenna *et al.*, 1998). Cells are plated on matrix-coated flexible substrates and are mechanically stretched by application of vacuum or air pressure, or by shaped inserts (Pender and McCulloch, 1991). The strain gradients created in substrate elongation systems are not uniform across the diameter of the dish, so if constancy of force levels is an important issue in experimental design and interpretation, these types of devices limit observations on cells to relatively small zones at a fixed radius from the center of the dish. Further, observing and measuring mechanoresponses in substrate elongation systems restricts the type of measurements that are made. This is particularly critical when the mechanoresponse may rapidly follow the force stimulus, such as an increase of $[Ca^{2+}]_i$. This limitation can be partly overcome for fluorescence dye measurements of ions if the force application system is physically integrated with the spectrofluorimetric measurement system (Arora *et al.*, 1994). Global mechanical stretching of the cell membrane can also be effected by inducing regulatory volume increase with hyposmotic buffers (Star *et al.*, 1992; Bibby and McCulloch, 1994) but the influence of the extracellular matrix on stretch-induced mechanical responses are not easy to quantify in these systems since forces are not applied through matrix adhesion sites but rather across the whole cell membrane.

On the basis of early measurements of the rheological properties of cytoplasm using internalized magnetite beads, a system of mechanical loading was developed (Glogauer *et al.*, 1995) in which cells that bound collagen-coated magnetite beads were placed within a magnetic field (Fig. 3). With this system the magnitude of loading can be controlled by the flux density of the external magnetic field and by the bead-loading density. Notably, coating of the magnetite beads with collagen permits binding of the beads to collagen receptors (mainly the $\alpha 2\beta 1$ integrin) and consequently, integrin-dependent mechanotransduction processes can be analyzed (Katsum *et al.*, 2004). Analysis by single-cell ratio fluorimetry of fura 2-loaded cells demonstrated large Ca^{2+} transients (50–300 nM above baseline) in response to magnetic force applications through collagen beads. Experiments using either the stretch-activated channel blocker gadolinium chloride or EGTA to chelate external Ca^{2+} ions, or addition of extracellular manganese ions, indicated that there was a Ca^{2+} influx through putative stretch-activated channels. The probability of a Ca^{2+} influx in single cells was increased by higher surface bead loading and the degree of cell spreading. Depolymerization of actin filaments by cytochalasin D increased the amplitude of Ca^{2+} response twofold. The regulation of Ca^{2+} flux by actin filament content indicated a possible modulatory role for the cytoskeleton in channel sensitivity. With this system, sufficiently large membrane distortions are induced to activate stretch-sensitive Ca^{2+}-permeable channels. Notably, the development and use of rotational bead models also provides excellent opportunities for study of mechanotransduction through matrix adhesion receptors and the cytoskeleton (Wang *et al.*, 1993), although this particular model has not been frequently applied to studies of mechanosensitive channel activation.

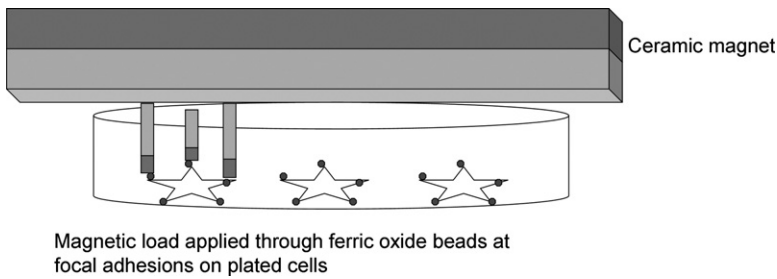

FIGURE 3 Simplified diagram of magnetic system for applying force to collagen-coated beads attached via integrins to the dorsal surfaces of cultured cells.

As noted above, cells in many connective tissues act in an integrated and collective manner for transducing mechanical forces into biochemical signals. Thus, forces delivered through both extracellular matrices and intercellular junctions can impact mechanotransduction processes (Ko and McCulloch, 2001). Cells in mechanically active, soft connective tissue environments such as the periodontal ligament (Beertsen and Everts, 1980) form extensive, cadherin-mediated intercellular junctions that are important in tissue remodeling and cell differentiation. For examination of cadherin-mediated force transmission in connective tissue cells, human gingival fibroblasts in suspension were plated on established homotypic monolayer cultures. The cells formed intercellular adherens junctions. Controlled mechanical forces were applied to intercellular junctions by electromagnets acting on cells containing internalized magnetite beads. At early but not later stages of intercellular attachment, force application visibly displaced magnetite bead-loaded cells and induced Ca^{2+} transients (65 $\pm$ 9 nm above baseline). Similar Ca^{2+} transients were induced by force application to anti-N-cadherin antibody-coated magnetite beads. Ca^{2+} responses depended on influx of extracellular Ca^{2+} through mechanosensitive channels because both Ca^{2+} chelation and gadolinium chloride abolished the response and manganese chloride quenched fura-2 fluorescence after force application. Force application induced accumulation of microinjected rhodamine-actin at intercellular contacts; actin assembly was inhibited by buffering intracellular Ca^{2+} fluxes. These results indicated that mechanical forces applied to intercellular junctions activate stretch-sensitive Ca^{2+}-permeable channels, increase actin polymerization and that N-cadherins in fibroblasts are evidently intercellular mechanotransducers (Ko *et al.*, 2001).

Elucidation of the nature of mechanosensors at a subcellular level has been advanced by tools developed through nanotechnologies. These tools include the use of innovative substrates with highly precise determination of the placement of size and locales of adherent proteins (Balaban *et al.*, 2001). Notably, recently developed tissue scaffolds have been produced which enable improved understanding of the mechanosensing, mechanotransduction, and response elements of force signaling (Vogel and Sheetz, 2006). Further advances include substrates with precisely determined rheological properties and the application of laser tweezers and atomic force microscopy by which operators can estimate the magnitude of the forces required to disrupt, for example, cell-matrix interactions (Jiang *et al.*, 2003; Zhang *et al.*, 2004). In combination with the development of technologies for isolation of mechanosensors associated with extracellular matrix adhesion receptors (Fig. 4), there is now hope for identifying what molecular components of adhesive cellular systems are involved in regulating

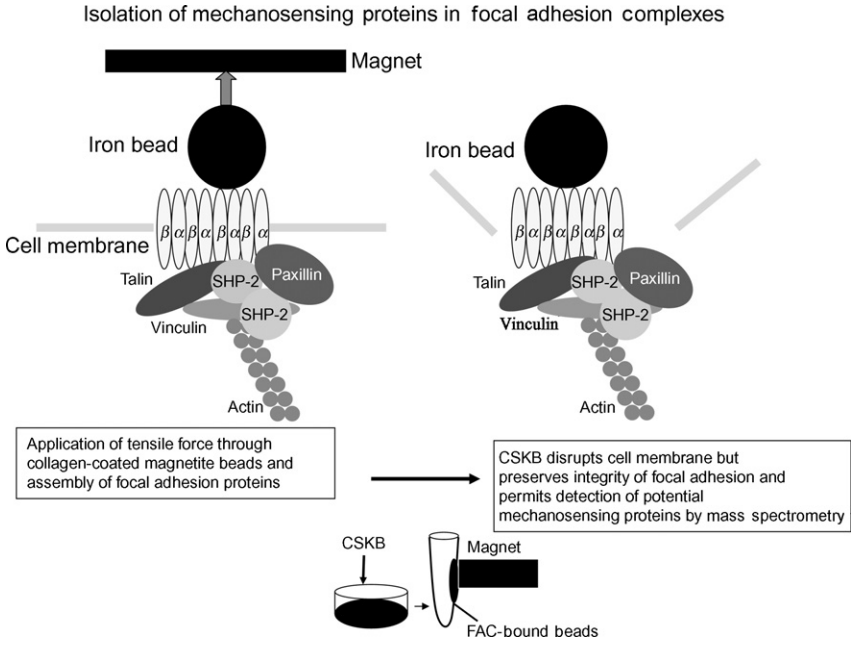

FIGURE 4 Method for purifying focal adhesion associated proteins following application of tensile forces to cultured cells. Panel on left shows removal of proteins from beads and their subsequent analysis by tandem mass spectrometry.

and determining the activation of mechanosensitive channels in connective tissues.

VII. EFFECTS OF FORCE ON CELL SURFACE STRUCTURES

If applied forces are of sufficiently high amplitude, alterations of protein folding and conformational changes at quaternary and tertiary structural levels can occur (Alberts *et al.*, 2002). As a result of force application, the properties of proteins can be considerably altered as new binding sites are uncovered and new folds are created at other sites in the molecule (Vogel and Sheetz, 2006). These changes in structure have important implications for information-rich molecules such as extracellular matrix proteins including collagen that are heavily endowed with cell attachment motifs, degradation initiation sites, and domains that impact the differentiation of cells that attach to these proteins. Conceivably, alterations of protein configuration

may be very early events at the initiation of mechanotransduction processes. In this context, we consider that mechanotransduction is a fundamental process by which mechanical signals are converted into biochemical outcomes. Nanotools have the capacity to reveal with considerable precision the sites where initial contacts between cells and matrix molecules take place and, possibly, where the mechanosensors are located. Nanotools may also be able to measure to within a few piconewtons, the amplitudes of force required to generate signals within cells (Jiang *et al.*, 2003). At cell-matrix interfaces, forces may be able to expose cryptic peptide sequences, permitting new receptor-ligand binding interactions, or strengthening preexisting interactions. Although not examined in any detail, changes in protein folding could result in altered conformation of mechanosensitive channels, thereby leading to activation or inactivation of channels. Notably, in addition to the exposure of cells to exogenous forces, cell-generated forces also exert effects on the surrounding matrix that have profound effects on tissue formation and maintenance and that are regulated by the rigidity of the substrate (Guo *et al.*, 2006).

VIII. FUTURE APPROACHES

This chapter explores the progress that has been made on the structure, function, and regulation of mechanosensitive channels. While connective tissues and their extracellular matrices are strongly impacted by mechanical forces as shown by the tight interdependence of mechanical loading and alteration of matrix structure/function, it is evident that our knowledge of mechanosensitive channels in connective tissues is rather meager. Further, the structural relationships between mechanosensitive channels and matrix adhesion receptors are only starting to be explored, in spite of the large amount of data showing functional connections. The advent of proteomic approaches that examine protein–protein interactions and particularly in those restricted to subcellular fractions suggests novel approaches to characterize channel-adhesion receptor and channel-matrix protein interactions. One possible approach for defining these interactions is shown in Fig. 4 in which focal adhesion proteins are isolated from cells with collagen-coated beads. The bead-associated proteins, with presumptive interacting channel proteins, can then be examined by tandem mass spectrometry and confirmations of potential interactions between adhesion receptors and channel proteins then examined by coimmunoprecipitations. The connective tissue-extracellular matrix-mechanoreceptor field is a subset of the global mechanotransduction arena which holds considerable promise for future studies. What is now needed are structural and functional definitions of how forces

directed along extracellular matrix proteins interact with and regulate mechanosensitive channels.

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