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Modelling the effects of vascular stress in mesangial cells

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It has recently been shown that mesangial cells are subjected to multiple forms of mechanical strain (fluid shear, hydrostatic pressure, and triaxial stretch) as a result of forces exerted by the vasculature. Nevertheless, the exact nature and the relative response to these stimuli have not been clarified. Although it is now well established that cyclic stretching of mesangial cells in culture results in the overproduction of extracellular matrix, indicating how intraglomerular hypertension may lead to glomerular scar formation, the contribution of different intracellular signalling mechanisms and extracellular mediators of the response are only now being identified. Recent studies point to a role for high glucose concentrations, transforming growth factor beta and its receptors, vascular endothelial growth factor, and connective tissue growth factor as important mediators, or modifiers of the response to mechanical strain. Although evidence exists for a role for protein kinase C, recent studies also implicate the mitogen-activated protein kinases along with enhanced DNA-binding activity of AP-1 as part of the signalling cascade altering matrix synthesis and cell proliferation in response to stretch. Finally, recent studies examining the effects of oscillating hyperbaric pressure demonstrate similarities, as well as differences, in comparison to those of cyclic stretch. Curr Opin Nephrol Hypertens 9:43–47.

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Abbreviations

CTGF	connective tissue growth factor
ECM	extracellular matrix
ERK	extracellular signal-regulated kinase
JNK	c-Jun NH_2-terminal kinase
MAPK	mitogen-activated protein kinase
MEK	mitogen-activated protein kinase or extracellular signal-regulated kinase
MC	mesangial cells
PTK	protein tyrosine kinase
PKC	protein kinase C
TGF-β	transforming growth factor beta
VEGF	vascular endothelial growth factor

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Introduction

Since the importance of glomerular hypertension as a causal factor in progressive renal sclerosis was first demonstrated, great interest has been shown in determining how this physical force is translated to the biochemical alterations characteristic of the disease [1–3]. Our laboratory [4] and that of Harris *et al.* [5] showed in 1992 that the application of cyclic stretch to mesangial cells (MC) in culture stimulated the production of extracellular matrix (ECM) molecules, suggesting how this might occur. Further relevance was indicated by our finding that the level of increased ECM secreted was directly related to the amplitude of the stretching force. These cells appear to be largely responsible for mesangial matrix synthesis *in situ*, and are therefore the likely contributors to the increased ECM deposition and expansion that characterize glomerulosclerosis [6]. In the intervening years, work in this field has focused primarily on three areas: (i) the nature of the mechanical force acting on MC; (ii) signalling events (primary and secondary); and (iii) soluble mediators or modifiers of the cellular response to mechanical force. This article will review work in the area, concentrating on advances made during the preceding year.

The nature of vascular forces acting on mesangial cells

Our understanding of the physical forces acting on the MC is still rudimentary. Nevertheless, three forms of mechanical stress are possible as forces exerted by the vasculature on MC: (i) fluid shear; (ii) hydrostatic pressure; and (iii) triaxial stretch. The first, laminar shear resulting from flow, and its changes (a function of both velocity and fluid viscosity), has been established as an important factor in capillary physiology. Within the capillary, the endothelial cell appears to act as a sensory cell, whereas the vessel smooth muscle cell functions as a responder cell [7]. However, evidence is currently lacking for similar effects of laminar shear on MC physiology. Plasma fluids pass from the capillary space through the mesangium, creating some level of shear, but the slow trafficking of this fluent would be expected to generate only a low force on the MC. The direct effect of shear force on MC has not been reported. However, a report that the supernatant of endothelial cells exposed to shear inhibits the proliferation of MC, suggests a possible indirect effect derived from this form of mechanical stress [8].

MC are subjected to vascular hydrostatic pressure as a result of the nature of glomerular basement membrane.

This membrane surrounds the entire mesangial lobule, but is discontinuous around individual capillaries. This allows the transfer of intracapillary pressure, and its fluctuations to the mesangium. It is expected, therefore, that the hydrostatic pressure within the mesangium is equal to that in the capillary [9]. As a result of the normal dampening of intraglomerular pressure at the afferent arteriole, it is not clear, however, what constitutes a pathological level of pressure. A limited number of studies have focused on this form of vascular stress and will be discussed below.

The third type of mechanical force acting on the MC is stretch. We have demonstrated that increased capillary pressure results in glomerular expansion as a result of the marked compliance of the structures involved [4]. The overall glomerular distention causes the outward movement of the paramesangial basement membrane to which MC cytoplasmic projections are firmly attached [10]. The consequence of pressure-induced glomerular distention is thus MC mechanical strain in the form of triaxial stretch. Glomerular distention can be extensive, with increases of up to 30% of basal volume [11]. However, under normal conditions, because glomeruli are exposed to only small pulse–pressure variations and not to the low frequency, moment-to-moment oscillations in systemic pressure, volume remains stable [12]. This tight control of intraglomerular pressure is the result of the very effective autoregulation provided by the afferent arteriole [13]. Such protection is characteristically impaired, however, in many models of progressive renal disease, including the diabetic and remnant kidney [13,14]; this results in wide swings in glomerular volume, magnified by arterial hypertension. Normal glomeruli with intact autoregulation vary their moment-to-moment volume by 0.4%, whereas those from remnant kidneys of hypertensive animals show variations of up to 7.3% [11]. For this reason, our laboratory has chosen to model the effects of mechanical strain *in vitro* using three cycles per minute, as opposed to other investigators who have chosen a rapid 60 cycles per minute, apparently to simulate forces generated by pulse pressure. Although we are beginning to understand the basic nature of the forces acting on the MC, much information is therefore still lacking.

Soluble mediators of the response to mechanical strain

Studies on the effects of cyclic strain on MC have shown a role for specific secreted cytokines or growth factors in the response. For example, we have shown that cyclic stretch of cultured MC upregulates the expression of transforming growth factor beta (TGF-β)1, a growth-regulating, prosclerotic cytokine [15]. In addition to an increase in the messenger RNA level, there is increased secretion and activation of the latent molecule. Furthermore, this response is specific because the activities of IL-1, TNF-α and even TGF-β2, remain unchanged [15]. The relevance of these findings is indicated by the now abundant evidence for TGF-β as a causal factor in various forms of glomerulosclerosis [16–19]. More recently, it was shown by Hirakata and colleagues [20] that stretch-induced mRNA expression of TGF-β occurs primarily by tyrosine kinase-dependent mechanisms, suggesting how this upregulation might occur.

The level of extracellular glucose appears to influence greatly the response to cyclic stretch. We have recently shown [21] that the induction of TGF-β activity and collagen accumulation by stretch is further enhanced in the presence of increased glucose concentrations. The marked increase in collagen accumulation that occurs under these conditions is reversed by the action of a TGF-β neutralizing antibody [21]. In support of our findings, Hori *et al.* [22] reported that anti-latent TGF-β binding protein antibodies or synthetic peptides corresponding to the N-terminal portion of anti-latent TGF-β binding protein type 1 could also inhibit the stretch-induced mRNA expression of type I collagen and fibronectin.

Riser *et al.* [15] also found that in cultures of MC exposed to a gradient of stretching amplitude, only those cells subjected to significant mechanical strain demonstrated intense immunostaining for the active form of TGF-β, as opposed to those in the same culture experiencing little or no strain. This differential localization of active TGF-β occurred even though the conditioned media bathing all cells contained greatly increased amounts of this cytokine, compared with unstretched control cultures. This suggested the possibility that TGF-β binding was increased in response to cyclic strain. In our most recent studies, we found that exposure to cyclic stretch significantly increased the overall number of TGF-β receptors as well as the ligand associated with TGF-β receptors (βR) I, II and III. βRI and βRII are the signalling receptors, whereas βRIII, or betaglycan, is a membrane protein that increases the binding of TGF-β1 and β3 to the signalling receptors and may be necessary for equivalent TGF-β2 binding [23]. Our finding of increased transcript levels and immunoreactive protein for TGF-β receptors, in the absence of a significant change in the apparent dissociation constant, indicated that stretch-induced binding was the result of increased receptor synthesis and expression, and was not due to a change in binding affinity [24•]. A similar but elevated response was obtained when MC were grown in high glucose-containing medium. In contrast, however, to the combined effects of high glucose and stretch on the induction of TGF-β secretion, no concerted or synergistic effect on receptor expression and binding was detected [24•]. This suggests that control mechanisms for the upregulation of the ligand, in

contrast to the receptor, may be distinct. Most importantly, these results indicate that the modulation of TGF-β receptors may be an additional control point in the mechanism of mechanical force-induced increase in ECM deposition by MC.

Another mediator that may play a role in the response of MC to mechanical strain is vascular endothelial growth factor (VEGF). A promoter of vascular permeability, VEGF is induced in MC by both TGF-β and stretching, and induces proteinuria in rats [25]. Gruden and coworkers [26•] recently investigated the effects on MC VEGF production of angiotensin II, the interaction between angiotensin II and stretch, and TGF-β blockade. Angiotensin II increased VEGF expression and production, whereas the angiotensin II receptor antagonist losartan prevented angiotensin II-induced, but not stretch-induced VEGF secretion, suggesting that different mechanisms were involved. Stretch-induced VEGF production was also unaffected by the addition of TGF-β neutralizing antibody, indicating that TGF-β was not involved. Finally, there was a significant additive effect on VEGF production when MC were pre-exposed to stretch then treated with angiotensin II. Although the effects on angiotensin II binding were not determined, qualitative protein analysis using immunoblotting suggested that angiotensin II receptor expression was upregulated in the prestretched MC [26•].

Most recently, a newly identified prosclerotic cytokine, connective tissue growth factor (CTGF) has been investigated in our laboratory as a possible element in the development of glomerulosclerosis, particularly that associated with diabetes. The potential importance of this cytokine was suggested by studies [27], which demonstrated in other cell types the induction of CTGF by TGF-β. First, we found that exposure of MC to recombinant CTGF markedly increased the secretion of fibronectin and collagen I [28•]. Second, MC were shown to express mRNA and secrete CTGF protein at relatively low levels. However, transcript levels and protein secretion were greatly upregulated by exposure to TGF-β and high glucose. Blockade studies with TGF-β neutralizing antibody demonstrated that the effect of high glucose was mediated by TGF-β. In another set of experiments, we found that cyclic mechanical strain markedly upregulated CTGF expression [28•]. This induction was first noted at 2 h of stretch and remained steady for the 48th observation period. This early induction suggested that stretch-induced CTGF expression might occur independent of TGF-β. Interestingly, however, the same level of stretch failed to increase the levels of secreted CTGF protein. The reason for this inconsistency is unknown. It may be that the culture conditions were not optimal for the expression of CTGF protein. Alternatively, the inability of newly synthesized CTGF to detach from the cell/matrix, or a simultaneous increase in CTGF catabolism may explain the observation. Although CTGF appears to play a role in MC metabolism of ECM, additional work will be required to define the role of mechanical strain in CTGF regulation. The in-vivo significance of these results was indicated by our finding that the glomeruli of mice, early in the development of diabetic nephropathy, demonstrate a 28-fold induction of CTGF transcript levels compared with control animals [28•].

Cell signalling and mechanical strain

The intracellular mechanisms of stretch-induced ECM production have not been elucidated. However, mechanical strain has been shown rapidly to activate both protein kinase C (PKC) and protein tyrosine kinase (PTK) in MC [20,29]. Akai *et al.* [29] reported that PKC activation is followed by the induction of mRNA for the AP-1 transcription factor *c-fos*, and inhibition of PKC blocks the increase. Changes in this transcription factor have been linked to increased ECM production. The TGF-β1 promoter also contains AP-1 consensus sequences and c-fos mediates autoinduction of the cytokine [30]. More recently, Ishida and coworkers [31•] showed that mitogen-activated protein kinase (MAPK) may also play a role in the overproduction of fibronectin in MC exposed to stretching. MAPK extracellular signal-regulated kinase (ERK) and c-Jun NH_2-terminal kinase (JNK), were activated by mechanical strain in a time- and intensity- (10–30% elongation) dependent manner. Significant increased activity occurred at 15%, or greater, elongation. Stretch-induced activation of ERK was inhibited by a PTK inhibitor, but not by inhibitors of PKC [31•]. Stretch also enhanced DNA-binding activity of AP-1, and this change as well as stretch-induced fibronectin production was blocked by an inhibitor of MAPK or ERK kinase (MEK). These results indicate that the activation of ERK may also mediate the overproduction of ECM proteins in MC exposed to mechanical strain. Another recent study aimed at identifying the signalling sequence(s) in the response to stretching was conducted by Ingram and colleagues [32•]. They reported that when MC were subjected to high levels of stretch (29% elongation) an early activation of MAPK, p44/42 and p38/HOG but not JNK occurred, and was followed by an induction of proliferation. In contrast, at a moderate level of stretch (20% elongation) there was a lesser increase in p44/42, no increases in p38/HOG or JNK, and no induction of cell proliferation. This indicated that the proliferation induced by mechanical strain is related to marked activation of MAPK, p44/42 and p38/HOG. The reason for the difference in JNK response in the two studies is not clear. However, we have observed that the conditions of culture, including cell density, serum concentration in the medium, and the stretching cycle, can

substantially influence the cell response. Both of these studies utilized low passage rat MC stretched at 60 cycles per minute. However, the latter study [32•] employed confluent cultures rendered quiescent by 24 h in medium containing 0.5% fetal calf serum. In contrast, the former study [31•] used subconfluent MC cultures made quiescent by incubation in medium containing 0.2% bovine serum albumin.

One important area that has remained largely unexplored is the transmission of the external mechanical force to an intracellular primary signal. Intuitively, the prominent actin cytoskeleton of MC, organized as stress fibers, has been proposed as an important transducer of mechanical stretch into excessive ECM. We noted that, as with other cells subjected to mechanical forces *in vitro*, stretched MC reshape their cell bodies and align their stress fibers in an orientation perpendicular to the direction of the stretching force. In our most recent experiments [33], exposure of MC to a high glucose concentration decreased their stress fiber number and thickness and rendered them more susceptible to disassembly by cytochalasin D. Furthermore, MC grown in a high glucose environment, treated with cytochalasin D, and subjected to cyclic stretch could not assume the expected realignment of their cell bodies and stress fibers. This lack of alteration was not associated with overall cellular injury, as shown by its reversibility upon the removal of cytochalasin D. Finally, the absence of stress fiber formation was accompanied by an enhanced fibronectin formation in the stretched MC. These results suggest that an organized array of stress fibers modulate the metabolic effects caused by mechanical force.

The response of mesangial cells to hyperbaric pressure

In comparison to mechanical stretch, far less is known about the MC response to pure pressure. Kawata *et al.* [34] used a pressure loading apparatus to subject MC to a constant high pressure, or in the case of controls, an atmospheric pressure. They showed that pressure enhanced G1/S progression and promoted the rate of DNA synthesis. The same group subsequently showed that a constant pressure load activated MAPK and induced the production of c-fos, a nuclear transcription factor [35]. An upstream MEK inhibitor of MAPK inhibited this induction. MAPK phosphorylation and cell proliferation by high pressure was significantly reduced by a PTK inhibitor, but not by PKC inhibitors. Antisense MAPK DNA inhibited MAPK expression by 80% in MC and significantly blocked pressure-induced cell proliferation, as did a MEK inhibitor. This indicated that pressure, an activator of MAPK, induces the activation PTK, and stimulates proliferation. Most recently, Mertens and colleagues [36] tested the effects cyclic pressure on MC growth and collagen synthesis using an oscillating pressure chamber. The authors found that in subconfluent cultures, oscillating high pressure increased neither cell- nor medium-associated collagen synthesis. However, in confluent MC cultures the same treatment resulted in increased medium-associated collagen. Exposure to high glucose increased mainly the cell-associated collagen fraction, which was further increased by oscillating pressure. The incubation of MC in high glucose concentrations stimulated cell proliferation, and 1–7 days of oscillating high pressure significantly decreased proliferation under both glucose conditions. The study showed that MC growth and collagen synthesis are influenced by hyperbaric oscillating pressure, supporting the theory that pure glomerular pressure plays a role in progressive glomerulosclerosis, inducing responses similar to those observed with cyclic stretching.

Conclusion

Triaxial stretch of MC results from glomerular hypertension, and has been modelled in cell culture using cycles that mimic either pulse pressure or moment-to-moment changes in systemic pressure. The effect of stretching amplitude has also been examined. Recent work to determine how such mechanical force leads to the overaccumulation of ECM has focused on signalling mechanisms and soluble mediators. Those studies provide evidence for complex signalling pathways beginning with a cytoskeletal response to force exerted on focal adhesions. In addition to the activation of PKC and PTK, there is enhanced activity of MAPK, p44/42, ERK and JNK, which appears to be intensity dependent and casually related to the overproduction of ECM proteins and altered MC proliferation. The upregulation of extracellular mediators and their receptors, such as TGF-β and CTGF appear also to play a role in stretch-induced ECM accumulation. These responses to stretch are highly influenced by the extracellular glucose concentration. CTGF appears to act downstream of TGF-β, but may be induced by stretch independent of TGF-β action. In addition to the effects on matrix accumulation, MC stretch may affect glomerular permeability through the induction of VEGF. This factor is upregulated by angiotensin II, TGF-β and stretch, although the mechanisms for stimulation appear to be different for each. Although in comparison with cyclic stretch, few studies have examined the effects of hyperbaric pressure on MC, recent work has indicated largely similar responses. This includes the activation of MAPK, altered proliferation and, under certain conditions, increased ECM secretion. Expanded studies in all of the above areas will be necessary if we are elucidate the complex pathways whereby mechanical strain produces the alterations characteristic of progressive glomerular disease. Such studies will require consideration of the cycle, amplitude and type of force applied, as well as the cell density or proliferative state.

Acknowledgements

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