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Abstract

Aims. Most benign bladder pathologies are associated with an increase of extracellular matrix (ECM – fibrosis) and may progress from formation of stiffer matrix to a more compliant structure. The aims were to summarise current knowledge of the origins of bladder fibrosis and consequences in bladder function.

Methods. A meeting at the International Consultation on Incontinence Research Society 2017 congress discussed the above aims and considered paradigms to reduce the extent of fibrosis. Discussants based their arguments on the basis of their own expertise, supplemented by review of the literature through PubMed. Proposals for future work were derived from the discussion

Results. Altered urodynamic compliance when ECM deposition is increased is mirrored by changes in the elastic modulus of isolated tissue, whether compliance is decreased or increased. No changes to compliance or fibrosis have been reported after botulinum toxin injections. Several paracrine and autocrine agents increase ECM deposition, the role of TGF-β was particularly emphasised. None of these agents has a net long-term effect on detrusor contractility and the reduction of contractile performance with increased ECM is due solely to a loss of detrusor mass. Several strategies to reduce fibrosis were described, ranging from potential therapeutic roles for vitamin-D or endostatin, manipulation of intracellular pathways that mediate myofibroblast differentiation and the potential role of the anti-fibrotic hormone relaxin. An understanding of epigenetic regulation of ECM deposition was also considered.

Conclusions. The conclusion that reduced bladder contractile function with increased fibrosis is due largely to the replacement of detrusor with ECM offers a way forward for future research to consider approaches that will restore bladder function by reducing ECM deposition.

Background

At the International Consultation on Incontinence Research Society (ICI-RS) in Bristol, UK, 2017, a panel of clinicians and scientists participated in a think-tank to review the consequences of increased fibrosis on bladder function and the identification of potentially useful therapeutic pathways to reverse the process. This review is a summary of the discussions and suggests directions to future research.

Extracellular matrix and fibrosis.

The extracellular matrix (ECM) is a non-cellular component of all tissues that provides a physical structure for the cellular components but also provides mechanical and chemical signals required for tissue growth and differentiation. It consists of fibrous proteins, such as collagens, elastins and fibronectins, and a ground substance of proteoglycans. Connective tissue includes ECM and associated cellular components, such as fibroblasts, and fibrosis refers to the excessive formation of connective tissue, say as part of a tissue repair or a reactive process.

Collagen is the most abundant ECM fibrous protein; its formation into fibrils provides tensile strength and the proteins themselves regulate other functions such as cell adhesion, cell migration and development[1]. There are numerous subtypes and collagen-I and –III are most important in determining biomechanical properties. Elastin is associated with collagen, which permits ECM to recoil after stretch, and fibronectin directs the organisation of ECM and mediates cell attachment. Proteoglycans consist of glycosaminoglycan (GAG) chains linked covalently to a protein core[2]. Matrices of these hydrophilic molecules are able to withstand high compressive forces.

ECM in a compliant tissue is formed of a relaxed network of collagen (type-I and –III), elastin and fibronectin embedded in a hydrogel of proteoglycans, secreted from non-activated fibroblasts, that can resist tensile as well as compressive forces. The actual composition is in a dynamic state dependent on external forces and cytokines and regulated by fibroblast secretion of matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs)[3].

Acute injury or release of cytokines due to physical or chemical stresses initiates fibroblastic responses to induce a type of wound-healing response. Injury itself initiates a fibrin clot and an inflammatory cascade characterised by transformation of attracted monocytes to macrophages. These in turn further secrete MMPs, growth factors and cytokines, as well as stimulate fibroblast migration and proliferation. The cascade progresses with the synthesis and deposition from fibroblasts of greater amounts of ECM proteins such as collagen type-I and –III, fibronectin and GAGs[4]. The deposition of large amount of ECM proteins increases local mechanical stresses induce the transformation or differentiation into myofibroblasts of cell types such fibroblasts, epithelial cells or circulating mesenchymal stem cells (see below). Myofibroblasts themselves have a much-increased capacity to synthesise and release ECM components, are themselves contractile and have the ability to promote the formation of large collagen bundles that together make tissue stiffer[5]. Continuation of the precipitating conditions can persist, for example in the bladder with increased bladder wall stress due to outlet obstruction or inflammation induced by ketamine. Consequently, continued ECM deposition and remodelling remain, increased myofibroblast numbers persist and TIMP production is greater than that of MMP to generate a fibrosis, a process possibly mediated by secretion of transforming growth factor-β1 (TGF-β1)[6,7].

Bladder pathologies: fibrosis, compliance and bladder wall stiffness.

An increase of fibrosis is associated with lower urinary tract disorders including: outflow tract obstruction[8]; spinal cord injury[9]; radiation-induced cystitis[10] or paediatric congenital anomalies[11]. In these pathologies fibrosis is evident in the spaces between muscle bundles and when it is more evident within bundles themselves. In addition, these conditions can often be associated with thickening of the lamina propria – between the urothelium and the detrusor – where there is also increased deposition of collagens, as well as an increase of interstitial cells that include fibroblasts and myofibroblasts[12].

Fibrosis might be expected to render the bladder wall stiffer, due to the deposition of greater amounts of collagen. Urodynamic measurements show that a decrease of filling compliance is associated with greater fibrosis on both adults[13,14] and children[15,16] in patients that have neurogenic disorders or outflow tract obstruction. Similar observations have been made in some animal models with similar pathologies[17,18]. However, decreased compliance is not a

ubiquitous phenomenon and several observations in humans and animal pathologies have demonstrated a distended, high compliance bladder[19-22]. It is not clear whether a low compliance phenotype precedes a more distensible, high compliance bladder or whether they develop along separate pathways. However, both are associated with an increased deposition of extracellular matrix. It has been noted by some of the above studies[20,21] that high compliance bladders are associated with poor contractile performance and it is possible that this contributes to the so-called underactive bladder phenotype.

Measurements of bladder compliance have been generally corroborated by more controlled biomechanical studies in isolated tissue strips or sheets. Under these conditions preparations are subjected to controlled rapid changes of length (strain) and the change to isometric tension (stress) recorded. With a tissue sample, such as the bladder wall, stress undergoes a partial relaxation, due to the relaxation of viscous components, whilst still undergoing the strain change. The elastic modulus, *E*, of the sample is the ratio of the change to steady-state stress for a given proportional change of strain. A stiffer substance has a higher value of *E* and may be regarded as being less distensible. This reciprocal relationship between elastic modulus and distensibility is reflected in an understanding of the term compliance, *C*, for a hollow structure such as the bladder, whereby C is the ratio of a change of volume, Δ*V*, for a change of internal pressure, Δ*P*; *ie*, $C=\Delta V/\Delta P$. Thus, there should be a reciprocal relationship between stiffness of bladder wall strips and the filling compliance of the bladder from where the strips were taken. This may be demonstrated, by data compared to control bladders and tissue, with: i) strips of low elastic modulus from bladders with high compliance (obstructed fetal sheep[19]); ii) strips of high elastic modulus from bladders with low compliance (exstrophy human bladder[11]); and iii) strips of normal elastic modulus from bladders with normal compliance (a subset of human obstructed bladders [A Wagg & CH Fry, unpublished data]).

An unresolved question is why bladder wall stiffness increases or decreases under different circumstances and it is unclear if the stiffer bladder represents an earlier stage in the process, compared to a late-stage more flaccid bladder. One observation is that with increased collagen deposition, many studies show that the subtype changes from one that is predominantly collagen-I to a greater proportion of collagen-III[23,24]. Increasing the type-III/type-I ratio of isolated collagen fibrils decreases their stiffness by up to ten-fold[25]. One explanation is that

compliance depends on the change of collagen ratio compared to the absolute increase of collagen. Another explanation is a variable deposition of proteoglycans, especially the extracellular small leucine-rich proteoglycans such as decorin, as these impart viscous properties without having great tensile stiffness[26]. An increase of proteoglycan deposition[20] would increase viscous relaxation during strain increases and therefore reduce the steady-state elasticity[27]. Thus, the biomechanical changes to a stiffer or more compliant direction will depend on the ratio of collagen to proteoglycan deposition. Furthermore, stress-strain relationships are not linear across all strains. A recent study with rats showed that at low strain levels the increase of stress is much smaller (a 'toe' region of the stress-strain relationship), which may be explained by initial re-alignment of collagen fibrils without development of significant stress, especially in the lamina propria[28]. At greater strains fibrils in the lamina propria and detrusor developed stress to demonstrate a more linear stress-strain relationship. This same study showed that bladder wall from aged animals showed an attenuated 'toe' region and thus would increase the overall slope of a stress-strain relationship. The reasons for this variable recruitment of collagen fibrils throughout the bladder wall deserves further study.

Botulinum toxin (BoNT/A) injections and bladder wall fibrosis.

A potential concern with repeated BoNT/A injections is an increase of fibrosis from the trauma of injection itself, or an action of BoNT/A itself or agents in the carriage medium. Children with neurogenic detrusor overactivity or with low compliance bladders did not have a greater ECM area after BoNT/A injection; and no inflammation or oedema[29]. A similar conclusion was reached in adults with idiopathic or neuropathic detrusor overactivity who received mucosal injection of BoNT/A; namely no change in suburothelium fibrosis, and where available in the detrusor layer, before and four or 16 weeks after BoNT/A injection[30]. Direct injection of BoNT/A into the wall of a bladder with neuropathic detrusor overactivity also caused no increase of fibrosis, compared to untreated bladders[31]. These observations are in harmony with experiments on rats wheren BoNT/A injections actually decreased fibrosis and reduced myofibroblast numbers[32].

Fibroblasts, myofibroblasts, connective tissue production and detrusor function.

The role of fibroblasts and myofibroblasts to produce and secrete structural and adhesive proteins as well as the ground substance that comprises ECM has been described above. It is of

value to understand the cellular pathways involved so that pathogenesis of excessive ECM deposition can be characterised and strategies developed to slow and reverse the process. Moreover, it is important to appreciate that fibroblasts control not just ECM deposition but other linked activity including wound healing, angiogenesis and recruitment of immune cells and the inflammatory response. Several paracrine agents promote fibroblast synthetic activity, including: platelet-derived growth factor (PDGF), interleukin-6 (IL-6) and eicosanoid leukotrienes[33]. Conversely, prostaglandin E_2 and prostacyclin (PGI_2) reduce the fibrotic response.

TGF-βs are the archetypal cytokines that promote fibrosis and are produced by other tissues but also fibroblasts themselves. They bind to serine/threonine kinase receptors on the cell surface that phosphorylate intracellular Smad2/3 transcription factors to induce collagen production and fibrocyte-myofibroblast differentiation[34]. IL-13, produced mainly by mast cells, stimulates TGF-β1 production and hence increased collagen production through fibroblast proliferation and transformation to myofibroblasts[35]. An associated pathway is mediated by *wnt*-like signal transduction. *wnt* is a paradigm signalling protein that regulates cell proliferation, differentiation and migration by activation of membrane frizzled receptors - members of the G protein-coupled receptor (GPCR) superfamily - and promotes inhibition of glycogen synthase kinase 3β. A canonical intracellular pathway thereafter prevents the degradation of the transcription factor β-catenin that in turn acts analogously to Smad2/3[36]. This pathway has been identified in the bladder[37] but its role in the induction and maintenance of fibrosis in benign bladder conditions is unclear. Of interest, other GPCRs, such as the angiotensin type-I and the endothelin 1 receptors, regulate myofibroblast differentiation and fibrosis.

Fibrosis of the bladder wall is associated with a reduction of detrusor contractile performance. One question that arises is whether this is due solely to the replacement of muscle with ECM or if it is exacerbated by reduced muscle contractility associated with the array of paracrine and autocrine factors produced by activated fibroblasts and myofibroblasts. This has been addressed in three ways using human detrusor from patients with urodynamically normal and from obstructed human bladders [Fry CH, Johal N, unpublished data]. Firstly, the isometric force of isolated strips in response to a contractile agonist was normalised to the cross-

sectional proportion of smooth muscle. In this case the contractile force was directly proportional to the cross-sectional area of muscle and identical in the two groups. Secondly, the maximum velocity of shortening of contractile elements in bladder strips was measured using a modification of the Hill plot[38] – an index of muscle contractility. In both cohorts the maximum velocity of shortening was statistically similar. Thirdly, intracellular Ca^{2+} responses to contractile agonists such as carbachol, ATP, high-KCl and caffeine were recorded in isolated myocytes, data from the two groups were again statistically similar. Thus, the reduction of contractile force measured in detrusor samples from fibrotic bladders was not associated with muscle failure, but was due solely to a replacement of muscle with ECM.

Strategies to reduce fibrosis in pathological bladders.

Several approaches have been developed to reduce the production of ECM by fibroblasts and/or limit the differentiation of fibroblasts to myofibroblasts. For example, the active form of vitamin-D (1,25-dihydroxy-vitamin D3) reduces type-I collagen production in hepatic cells[39] and reduced fibrosis in other tissues such as lung parenchyma. Furthermore, vitamin-D3 deficiency is associated with liver fibrosis[40]. Endostatin is a small peptide derived from the carboxy-terminus of collagen type-XVIII and released by fibroblasts. The carboxy-terminal has antifibrotic properties (the amino-terminal inhibits angiogenesis) possibly via the TGF-β1 pathway and has been tested in lung and skin tissues[41].

Another strategic approach is to limit myofibroblast generation, a contractile phenotype characterised by expression of α-smooth muscle actin, a greater capacity for collagen secretion and a resistance to apoptosis. Several cells types act as sources for myofibroblast generation that include fibroblasts themselves; but also epithelial and endothelial cells; vascular smooth muscle cells; pericytes; and circulating blood cells such as monocytes and fibrocytes. These transitions are controlled by several developmental signalling pathways mediated by factors such as wnt, Sonic hedgehog and Notch ligands. Space does not enable detailed description of these pathways here but induction of bladder fibrosis from benign conditions offers an attractive approach that requires more scrutiny[42,43].

Epigenetics studies changes to gene function that do not involve changes to the DNA sequence. This suggests that epigenetic modifications do not alter the DNA sequence itself which suggests

they are reversible interventions and thus offer useful therapies to reduce fibrosis. Such modifications include histone post-translational modifications and DNA methylation events. For example, myofibroblast differentiation stimulated by TGF-β is mediated by histone deacetylase 4 and offers an attractive target[44]. Reduced histone acetylation attenuates the expression of cyclo-oxygenase-2, an enzyme that generates antifibrotic prostaglandins[45].

There is increasing interest in the role of the hormone relaxin as an agent to reverse fibrosis. This peptide hormone is part of the insulin superfamily whose production is increased during pregnancy and among other roles relaxes pelvic ligaments, softens the pubic symphysis and has a general antifibrotic action. There are eight peptide members (relaxin-1 to -4 and insulin-like-peptide-3 to -6) and four receptors (RXFP1 to RXFP4). Relaxin-2 is best characterized and binds to RXFP1, a G-protein coupled receptor that increases intracellular cAMP[46]. The final intracellular pathways require clarification but relaxin treatment increases MMP expression and downregulates TIMP expression. There is evidence that this involves the inhibition of the TGF-β1/pSmad2-induced increase of myofibroblast differentiation[47]. Serelaxin is a recombinant form of human relaxin-2 that has been trialled as an antifibrotic agent. It has been successful in human trials concerned with renal dysfunction in cirrhosis[48] and shows promise for other conditions such as pulmonary fibrosis[49]. No trials have been carried out in the bladder but a brief report demonstrated that effectiveness of relaxin to reverse fibrosis from exposure to X-rays to restore normal cystometric function[50].

Conclusions

An increase of extracellular matrix (ECM) is a feature of bladder wall morphology that accompanies many benign lower urinary tract conditions. One feature is a decrease in bladder compliance which is mirrored by an alteration in tissue stiffness. Collagen deposition generally has detrimental consequences on the active and passive contractile properties of the bladder wall. Thus, paradigms to reduce ECM deposition would be therapeutically attractive. Several possibilities exist, that require detailed examination *in vitro* and *in vivo*, ranging from the use of antifibrotic agents, manipulation of intracellular pathways that mediate altered expression and translation of ECM components, through to epigenetic manipulation of the genome.

Research questions

1. Measure the composition of ECM in terms of collagen subtypes and proteoglycans in relation to the stress-strain properties of bladder wall tissue.
2. Characterise the TGF-$\beta 1$ and *wnt*-signalling pathways in detrusor and lamina propria layers.
3. Determine the effectiveness of anti-fibrotic strategies to reduce bladder wall fibrosis.

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