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VASCULAR BIOLOGY(RS ROSENSON, SECTION EDITOR)

The Endothelium in Diabetic Nephropathy

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Abstract Diabetes is characterised by widespread endothelial cell dysfunction that underlies the development of both the micro- and macrovascular complications of the disease, including nephropathy, cardiomyopathy, and non-proliferative retinopathy. In the kidney, major changes are noted in glomerular endothelial cell structure in their fenestrations and glycocalyx. These changes, along with endothelial cell loss and capillary rarefaction in both the glomerulus and tubulointerstitium, lead to the progressive loss of glomerular filtration that render diabetes the most common cause of end-stage renal disease in much of the developed world. New treatments in diabetes that directly address the abnormal structure and function of the endothelial cell are desperately needed.

Keywords Endothelial · Diabetes · Glycocalyx · Proteoglycans · Fenestrae · Glomerular filtration · Fibrosis · Ischaemia · Hypoperfusion · Capillary rarefaction · Endothelial-mesenchymal transition · Vascular endothelial growth factor · Transforming growth factor-ß

Introduction

The capillary network is essential for providing an adequate supply of oxygen and nutrients. In the kidney, however, it has an additional *raison d'être* – the ultrafiltration of plasma. Accordingly, dysfunction or loss of glomerular capillaries, as is seen in diseases such as diabetes, cause a progressive reduction in glomerular filtration rate that in many cases leads to end-stage renal disease requiring dialysis or transplantation to preserve life.

As might be expected from the specialised function of the kidney's capillaries, most of the attention has focused on the glomerular endothelial cell that serves as the initial barrier to the transcapillary passage of cells and macromolecules into Bowman's space, while permitting the relatively unrestricted movement of water and solutes. In providing this function, the glomerular endothelial cell is notable for its cytoplasmic fenestrations, surface glycocalyx, and close proximity to podocytes with which it interacts in a bidirectional manner [1•].

Fenestrae

In organs such as the heart, skeletal muscle, and skin, the endothelial layer is continuous. In contrast, endothelial cells of the glomerulus are characterised by an unusually high abundance of trans-cytoplasmic holes or fenestrae that constitute 20–50 % of the cell surface and play a key role in the filtration of water and small solutes across the capillary wall [2].

Glomerular endothelial cell fenestrae are typically 70–100 nm in diameter in humans, and as such are easily visualised by electron microscopy [3]. They are not uniformly distributed but are localised to the thin, flat attenuated regions of the endothelial cell adjacent to neighbouring podocytes with which they share a common basement membrane [4]. At the hilum of the capillary loop, however, where the endothelial cell comes into contact with a mesangial cell rather than a podocyte, it is thicker and unfenestrated [5].

In a detailed study of glomerular ultrastructure, Toyoda and colleagues found that the fraction of the glomerular capillary luminal surface covered by fenestrated endothelium [S_s(Fenestrated/cap)] was reduced in individuals with type 1

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diabetes when compared with control subjects [6]. While no difference in S_s(Fenestrated/cap) was noted between normo- (32 %) and microalbuminuric (32 %) subjects, a further reduction was evident in those with macroalbuminuria (25 %) as compared with controls (41 %). Notably, these changes in endothelial cell structure paralleled changes in podocytes and in the mesangium such that S_s(Fenestrated/cap) correlated with mesangial fractional volume/glomerulus (r=-0.57, p=0.01), glomerular basement membrane surface with non-detached podocyte foot processes (r=0.61, p=0.007), and podocyte foot process width (r=-0.58, p=0.01) [6].

More recently, Weill et al. reported their studies of kidney ultrastructure in Pima Indians with type 2 diabetes [7•]. Similar to findings in type 1 diabetes, reductions in the proportion of fenestrated endothelium were noted in normo- (27.4 %), micro- (27.2 %), and macroalbuminuric subjects (19.3 %) when compared with donor biopsies (43.5 %). Moreover, clear correlations were evident between the proportion of fenestrated endothelium and the extent of both albuminuria and GFR. Notably, while podocyte detachment also correlated with albuminuria, it did not correlate with GFR [7•].

Although the biology of fenestra formation is not completely understood, substantial evidence indicates a pivotal role for vascular endothelial growth factor (VEGF) in their generation and maintenance [2, 8]. Pharmacological inhibition of VEGF signalling, for instance, induces loss of endothelial cell fenestrations and kidney dysfunction [9], as does the presence of high circulating levels of soluble VEGF receptor-1/fms-like tyrosine kinase 1 (VEGFR-1/flt-1) in pre-eclampsia [10].

Consistent with these findings, the diminution in VEGF expression as a consequence of podocyte loss in diabetes [11] may well explain the reduction in fenestrations in advancing diabetic nephropathy.

Glycocalyx

With a diameter of 70–100 nm in humans [3], the physical structure of the fenestrae alone would seemingly provide little impediment to the permeation of 3.6-nm diameter albumin, yet under normal circumstances albumin excretion is minimal. While the glomerular basement membrane and podocytes undoubtedly provide major barriers to the transglomerular passage of macromolecules, components of the endothelial cell also contribute. Indeed, recent attention has been focussed on the endothelial surface layer (ESL), not only for its role in anticoagulation and angiogenesis, but also for providing a key component of the glomerular barrier that contributes to the charge selectivity of the glomerulus through its abundance of negatively charged glycoproteins and proteoglycan. However, because the ESL is mostly removed during fixation and staining, it has been difficult to visualise, with measurements of thickness reported between 50 nm and 1 μm, depending upon the technique used [12]. Part of this wide disparity in size estimates may reflect, at least in part, the thickness of each of the two components of ESL: a surface glycocalyx that consists of proteoglycans with their glycosaminoglycan chains covalently bound to the cell surface, and a more loosely associated cell coat that is attached to the glycocalyx through charge-charge interactions [13].

Heparan sulphate is the most abundant glycosaminoglycan within the glycocalyx, although substantial quantities of other negatively-charged glycosaminoglycans, such as chondroitin sulphate and hyaluronic acid, are also present. Together, these macromolecules – and particularly the heparan sulphate side chain of its proteoglycan – were previously thought to account primarily for the charge selectivity of the glomerular filtration barrier. This assertion was based on key experimental findings that induction of proteinuria followed the administration of glycosaminoglycan-degrading enzymes [14] and anti-heparan sulphate antibodies [15]. Indeed, an inverse relationship between proteinuria and heparan sulphate-associated anionic sites in the glomerular basement membrane was noted in experimental diabetic nephropathy and other models of proteinuric kidney disease [16, 17].

More recently, the role of heparan sulphate-associated charge selectivity as a casual factor in the development of proteinuria has been severely challenged by findings in genetically engineered mice that do not develop significant albuminuria despite deficiency of heparan sulphate proteoglycan and associated diminution in anionic sites [18, 19]. However, while these findings do indicate that heparan sulphate may not have a primary role in determining charge selectivity during development, they do not discount the possibility that its modulation in the disease setting may have pathophysiological consequences. For instance, mice that lack the heparan sulphate-degrading enzyme heparanase do not develop proteinuria or structural injury when diabetes is induced with streptozotocin [20••]. This finding may be particularly significant given the induction of heparanase that is also noted to occur in proteinuric disease states, including diabetic nephropathy [21], where its overexpression may be induced by high glucose-induced production of reactive oxygen species and angiotensin II [22, 23].

From a therapeutic standpoint, specific treatments beyond glycaemic control and blockade of the renin-angiotensin system that restore heparan sulphate may be helpful. To this end, two approaches have been undertaken. Firstly, attempts to replenish lost charge by the administration of glycosaminoglycans have chiefly centred on sulodexide, a mixture of four glycosaminoglycan polysaccharides initially isolated from porcine lung and liver. Animal studies and smaller human studies using this approach have been encouraging. However, a large multicentre study in patients with type 2 diabetes, renal impairment, and significant proteinuria who were already

receiving maximal therapy with angiotensin II receptor blockers failed to show improvement in either decline in GFR or proteinuria with sulodexide versus placebo [24]. Similarly disappointing results were noted in a large study of sulodexide in microalbuminuric type 2 diabetic subjects [25]. An important caveat acknowledged by the study investigators was their inability to determine whether the administered sulodexide was absorbed from the gastrointestinal tract.

As an alternative to the administration of glycosaminoglycans, other investigators have focussed on strategies to diminish their degradation. Here, proof-of-concept studies have shown a diminution in proteinuria with a polyclonal anti-heparanase antibody [26, 27]. More recently, studies with SST0001 (sigma-tau Research Switzerland SA), a non-anticoagulant heparin with anti-heparanase activity, has been shown to reduce albuminuria and kidney injury in diabetic mice [20••]. However, given the role of heparanase in promoting myeloma growth, dissemination, and angiogenesis, current clinical trials with this agent have targeted multiple myeloma rather than kidney disease (NCT01764880).

While much glycocalyx research has focussed on its role in the glomerulus, it is prudent to recall that glycocalyx is not confined to the kidney, and is present on the luminal surfaces of endothelial cells throughout the vasculature. Moreover, although micro- and macroalbuminuria reflect the transglomerular passage of albumin, the excessive permeation of macromolecules occurs throughout the capillary network in diabetes, serving as a risk marker for proliferative diabetic retinopathy and macrovascular disease [28]. With these findings in mind, it is notable that the systemic volume of glycocalyx is reduced in both type 2 [29] and type 1 diabetes where it coincides with the development of microalbuminuria [30].

Glomerular Capillary Loss

Mesangial expansion is a characteristic feature of diabetic nephropathy that correlates inversely with the capillary filtering surface area in renal biopsies from patients with type 1 diabetes [31]. Since filtration is a function of the capillary filtering surface area, its loss leads to a reduction in GFR. What is unclear, however, is which comes first: capillary loss or mesangial expansion. From a mesangiocentric perspective, the occupation of additional space within the confines of the glomerular tuft would restrict the vascular surface area by a mass effect. Alternatively, glomerular capillary cell loss in diabetes may precipitate extracellular matrix expansion with the release of profibrotic factors by apoptosing endothelial cells [32].

Because endothelial cells do not require insulin for glucose entry, hyperglycaemia will lead to an increase in intracellular glucose and activation of its downstream pathways. One consequence of this increased metabolic flux is the elaboration of increased amounts of reactive oxygen species (ROS), which in turn lead to caspase 3-induced endothelial cell apoptosis via a range of intermediaries that include activation of NF-κB, c-Jun NH2 terminal kinase (JNK), and p38 MAP kinase, along with translocation of Bax and downregulation of Bcl-2 [33–36]. Other studies have indicated that in addition to ROS overproduction, glucose induction of TNF-α and TNF-α receptor expression may also induce caspase 3/7 apoptosis via activation of the transcription factor FOXO1 [37, 38]. Finally, non-glycaemic components of the diabetic milieu such as angiotensin II may also contribute to endothelial cell apoptosis [39] and may, in part, account for the renoprotective effects of renin-angiotensin system blockade.

Tubulointerstitial Capillary Loss

Although the glomerulus has been the predominant focus in studies exploring the structural changes of diabetic nephropathy, more attention is now shifting to the hitherto neglected tubulointerstitial component of the disease. As with the glomerulus, interstitial fibrosis and peritubular capillary loss are also major features of diabetic nephropathy. Indeed, similar to the relationship between GFR and glomerular capillary loss, the extent of peritubular capillary rarefaction correlates closely with declining kidney function in diabetes, as in other forms of chronic kidney disease [40].

Moreover, glomerular and peritubular loss are not only intimately related to each other, but also explain the pathogenesis of progressive fibrosis and inflammatory cell infiltration that characterise progressive kidney disease [41]. This interrelationship forms the basis of the "chronic hypoxia theory" put forth by Fine et al., whereby primary glomerular injury with capillary loss leads to diminished post-glomerular capillary flow, and consequently to tubulointerstitial hypoxia [41]. Deprived of sufficient oxygen and nutrients, the hypoxic tubular epithelial cells apoptose, stimulate inflammatory cell infiltrate, and initiate a fibrogenic response by stimulating myofibroblast formation. The resulting fibrosis causes capillary rarefaction, exacerbating the tubular atrophy that impairs the function of previously uninvolved glomeruli, creating a vicious circle of progressive disease.

Paracrine Signalling and Endothelial Cell Survival

In light of the pivotal role of the endothelium in ensuring the adequate delivery of nutrients and oxygen, it comes as no surprise to learn that a range of factors, particularly those secreted by nearby cells, assists in maintaining endothelial health. Their significance, however, is especially manifest in the disease state when the function and viability of the microvasculature is threatened so that the balance between pro- and

anti-angiogenic signals will ultimately determine the fate of the endothelium [42].

Vascular Endothelial Growth Factor (VEGF)

Of the various forms of VEGF and cognate receptors, most of the attention has focussed on VEGF-A and its type 2 receptor (VEGFR-2). Much of our understanding of the role of VEGF in diabetic nephropathy and kidney disease has come from work in animal models, many of which have focused on the role of VEGF in the induction of albuminuria. Here we will examine the effects of VEGF on endothelial cell viability and function, focusing on its role in the preservation of GFR rather than its effect on albuminuria.

In the rat, VEGF mRNA and protein are found in podocytes and in the epithelium of tubules and collecting ducts. VEGFR-2 expression, on the other hand, is predominantly localised to glomerular and peritubular capillary endothelial cells. Induction of streptozotocin-induced diabetes in the Sprague Dawley rat leads to an ostensibly protective overexpression of VEGF [43] such that, unlike that in humans, diabetes in this rat model is not accompanied by capillary rarefaction or decline in GFR.

A similar scenario of stress-induced VEGF upregulation has also been reported in the setting of hypertension. Here, hypertensive transgenic (mRen-2)27 rats that overexpress renin display a twofold increase in glomerular VEGF mRNA when compared with normotensive Sprague Dawley rats. The spontaneously hypertensive rat (SHR), which develops less kidney injury, displays VEGF expression that is intermediate between normotensive Sprague Dawley and severely hypertensive (mRen-2)27 rats [9]. Consistent with the notion of VEGF upregulation in response to endothelial stress, loss of its protective effects becomes more apparent in disease states where pharmacological inhibition of the VEGFR-2 kinase or knockout of VEGF expression leads to endothelial cell loss, glomerular injury, and decline in GFR [9, 44••, 45].

In humans, diabetic nephropathy is associated with progressive podocyte loss and tubular atrophy [11, 46]. Accordingly, a reduction in VEGF expression akin to that seen in the remnant kidney model may be anticipated. Indeed, this appears to be the case with reductions in both glomerular and interstitial VEGF seen in human diabetic nephropathy [47, 48].

Podocytes elaborate not only VEGF but a wide range of other factors that are thought to modulate endothelial cell structure and function, including angiopoietins 1 and 2, secreted protein acidic and rich in cysteine (SPARC), and stromal cell-derived factor-1 α (SDF-1α) [49, 50]. Accordingly, the model of kidney disease chosen to study their effects is critical in translating the findings of animal studies into human context, and while meticulous and painstakingly detailed studies of glomerular podocyte density have been conducted in biopsies from individuals with type 1 and type 2 diabetes, to our knowledge, comparable studies have not been undertaken in rodent models of diabetes.

Endothelial Cell Replacement: Role of Bone Marrow-Derived Angiogenic Cells

Until recently, endothelial cell replacement was viewed as a local phenomenon whereby neighbouring endothelial cells would proliferate and migrate to replace those that had been lost. However, in the past 15 years, a new paradigm has evolved whereby circulating pro-angiogenic cells derived from the bone marrow are now thought to be the predominant mechanism underlying microvascular repair in the adult. While an increase in these circulating pro-angiogenic cells, often referred to as EPCs, may be thought to compensate for the endothelial cell loss seen in diabetic nephropathy, the opposite seems to occur. When compared with age-matched controls, patients with both type 1 and type 2 diabetes have fewer circulating EPCs [51–53]. The reduction in cell counts is even more apparent in nephropathy, where diabetic subjects with microalbuminuria were found to have fewer circulating EPCs than normoalbuminuric type 1 diabetic patients, despite similar age, diabetes duration, and HbA1c [54]. Moreover, these reductions were also associated with a greater likelihood of nephropathy progression in patients with type 2 diabetes [55].

Compounding the effects of the reduction in EPC numbers, diabetes is also associated with dysfunction in the ability of these cells to proliferate, migrate, and stimulate angiogenesis in both the cell culture and in vivo angiogenic settings [53, 56–58]. Although the mechanisms that underlie this dysfunction are not clearly understood, some interesting parallels are emerging. As with diabetes, ageing is also characterised by

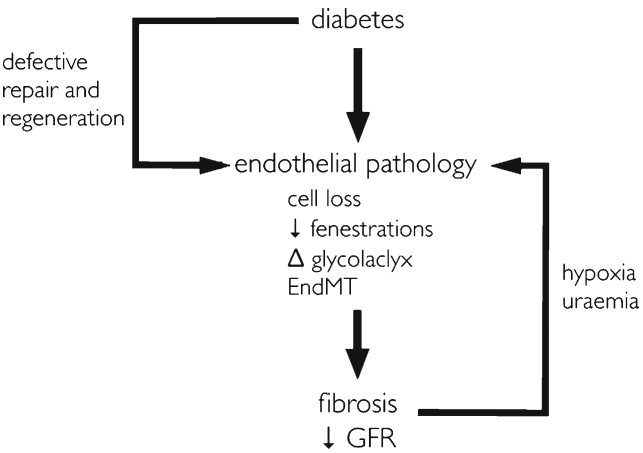

Fig. 1 The diabetic state induces several changes in endothelial cell structure and function that lead to progressive fibrosis, reduced capillary perfusion and diminished GFR. The resulting ischaemia exacerbates these changes and leads to a vicious cycle of further endothelial cell injury and worsening tissue hypoxia, all in the setting of a diminished capacity for endothelial cell repair and regeneration

microvascular loss and EPC dysfunction. With this in mind, our group hypothesised that silent information regulator protein 1 (SIRT1), a deacetylase implicated in the life-extending properties of caloric restriction and regulation of angiogenesis [59], may be a contributor. Consistent with the concept of metabolic memory [60], prolonged 7-day culture in normal glucose failed to restore the angiogenicity of EPCs derived from diabetic animals. This diabetes-associated defect was accompanied by a marked reduction in SIRT1 expression and diminished secretion of pro-angiogenic factors. Pharmacological SIRT1 activation restored both the production of these pro-angiogenic factors and their angiogenic activity [61], raising the tantalizing possibility of ameliorating microvascular loss in diabetes through the rejuvenation of EPCs.

Endothelial-Mesenchymal Transition

The phenomenon of epithelial-mesenchymal transition (EMT), whereby epithelial cells begin to express the characteristics of mesenchymal cells, has been established for some time. More recently, a similar change in cell phenotype, referred to as endothelial-mesenchymal transition (EndMT), has been observed in endothelial cells. Under the influence of a range of factors, endothelial cells begin to resemble myofibroblasts, with loss of polarity, diminished basement membrane adhesion, and the acquisition of mesenchymal markers such as fibroblast-specific protein 1 (FSP1), α-smooth muscle actin (αSMA), vimentin, and nestin, many of which are characteristic of active myofibroblasts [62].

Evidence of the involvement of EndMT in diabetic nephropathy may be found in the pivotal studies of Zeisberg [63] and Li [64], which have shown that 40–50 % of FSP1/αSMA co-express the endothelial cell marker CD31, and that this phenotypic switch occurs very early in the disease process. While the molecular mechanisms accounting for such EndMT are not completely understood, many of the factors known to increase as part of the diabetic milieu have been implicated, including transforming growth factor-ß, angiotensin II, and advanced glycation end products [62].

Clinical Trials

Current treatment for diabetic nephropathy centres on the use of agents that block the renin-angiotensin system (RAS). In addition to lowering systemic and intraglomerular pressures, angiotensin-converting enzyme inhibitors and angiotensin receptor blockers have also been shown to improve kidney capillary density in animal models [45]. Notably, such agents also exert anti-fibrotic effects that may explain, at least in part, the ability of RAS blockade to preserve the kidney's microvasculature [65]. As such, other agents with anti-fibrotic activity may also provide endothelial protection [66]. More recently, preclinical studies have focussed on the potential of cell-based therapies to protect and attenuate microvascular loss in the setting of kidney disease [67, 68]. A clinical trial using angiogenic mesenchymal precursor cells in advanced diabetic nephropathy is currently underway (NCT01843387).

Conclusion

In many ways, diabetic nephropathy may be viewed as a disease of the endothelial cell. The diabetic state induces several changes in endothelial cell structure and function that lead to progressive fibrosis, reduced capillary perfusion, and diminished GFR (Fig. 1). The resulting ischaemia exacerbates these changes and leads to a vicious circle of further endothelial cell injury and worsening tissue hypoxia, all in the setting of diminished capacity for endothelial cell repair and regeneration. Strategies that reduce endothelial cell loss and dysfunction, along with new ways to replace them, provide an opportunity to slow, arrest, or perhaps even reverse the progressive loss of glomerular filtration that characterises this devastating and all-too-common disease [67, 69, 70].

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Compliance with Ethics Guidelines

Conflict of Interest Richard E. Gilbert is a founder and shareholder in Fibrotech Therapeutics Pty Ltd., and has received consulting fees from Mesoblast Ltd. and honoraria for lectures and research grants from AstraZeneca, Bristol-Myers Squibb, and Merck.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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