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Renal tubular basement membrane and collagen type IV in diabetes mellitus

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Renal tubular basement membrane and collagen type IV in diabetes mellitus. The pathogenesis of the multiple structural lesions in diabetic nephropathy remains debated, and likely is multifactorial. The uniform thickening of the renal basement membranes lining the glomerular and tubular elements appears to be a consequence of the metabolic perturbations which are directly related to hyperglycemia. While most investigations have focused on the increased accumulation of extracellular matrix in the glomerular basement membrane and the mesangium, and their relation to derangements in glomerular function, little is known regarding the pathogenesis and the significance of the tubulointerstitial changes and the thickened tubular basement membrane (TBM). It is possible that these latter changes are causally related to the cellular hypertrophy of the renal tubular epithelium that lines the TBM. It has been postulated that in the earlier stages of the disease, hyperglycemia induces renal tubular hypertrophy and stimulates the synthesis of the various matrix components which are normal constituents of the TBM. Later, the structural composition of the TBM is susceptible to further modifications by non-enzymatic glycation, and this aberrant process may impart a relative resistance to matrix degradation leading to a slow turnover. In vitro investigations on murine proximal tubule cells in culture have provided evidence that elevated ambient glucose is a sufficient stimulus for cellular hypertrophy and increased biosynthesis of collagen type IV, the predominant constituent of TBM. High glucose levels increase steady-state collagen IV mRNA, partly due to transcriptional activation of *cis*-acting elements of the gene which are controlled by putative glucose-responsive *trans*-acting proteins. This effect of hyperglycemia may be the consequence of increased activity of the polyol pathway, with attendant alterations in cellular *myo*-inositol metabolism. Treatment with sorbinil, an aldose reductase inhibitor, or supplementation of the medium with supra-physiologic levels of *myo*-inositol, prevents the stimulation by high glucose of the increased secretion and biosynthesis of collagen IV. The glucose-induced cellular hypertrophy, however, is minimally affected by these maneuvers, and may result from activation of humoral or local mediators including transforming growth factor-β.

In considering the structural lesions which characterize the nephropathy of diabetes mellitus, much attention has been focused on the glomerulus. However, and as was recently reviewed [1], the contribution of non-glomerular lesions to the clinical spectrum of diabetic nephropathy has been much less appreciated, although careful studies have established that tubulointerstitial fibrosis [2, 3] and renal arteriosclerosis [4, 5] are important factors in the development of ischemic or obliterative glomerulosclerosis. A full understanding of the mechanisms that culminate in irreversible kidney failure requires a closer look at the status of the tubulointerstitium in diabetes mellitus.

Nephromegaly is also an early feature of the involvement of the kidney in diabetes mellitus and is predominantly reflective of increased renal tubule mass, mostly due to cellular hypertrophy of the tubulo-epithelium [6–8]. The importance of this lesion in the development of abnormalities in kidney function remains unsettled; it is speculated that the hypertrophied cell provides a priming factor for the initiation or maintenance of a fibrosing process in the tubulointerstitial compartment [1]. This is somewhat analogous to the relationship between diabetic glomerular hypertrophy and the development of glomerulosclerosis [9].

Within the broader context of examining the pathobiology of the renal tubulointerstitium in diabetes, this review will specifically focus on the status of the tubular basement membrane (TBM). Available data on the morphology and composition of this structure will be presented. The metabolism of collagen type IV, the most abundant constituent of the TBM, will be reviewed. Evidence is provided relating the central influence of elevated ambient glucose on renal growth and extracellular matrix production, with a consideration of the potential pathogenetic mechanisms.

Early TBM changes in diabetes and the relation to tubular cell hypertrophy

The abnormalities of the TBM in diabetes may be conveniently divided into two varieties: In the early phase of the disease there appears to be an acute increase in TBM mass which accompanies the development of renal hypertrophy; this is followed by the conspicuous thickening of the TBM which does not become apparent until a few years have elapsed.

The early changes in TBM mass are best appreciated by considering the induction phase of experimental diabetes. Seyer-Hansen, Hansen and Gunderson [7] examined rat kidney growth in streptozotocin-induced diabetes by morphometric analysis of various anatomical structures at different intervals after the onset of the disease. After only four days, proximal tubule cell volume and epithelial cell height were significantly increased (by at least 20%). This was later accompanied by significant increases in tubule length and luminal diameter. It is reasonable to assume that TBM dimensions, particularly the surface area, must have also increased as an accompaniment to acute renal tubular hypertrophy. Moreover, it can be argued that, triggered by some of the anabolic events that are restricted to the kidney, a substantial acceleration in TBM synthesis must take place so as to enlarge TBM area (or volume). This argument is analogous to that previously made by Osterby and

Gundersen [10] in their study on the fast accumulation of glomerular basement membrane (GBM) material in experimental diabetes that an acute increase in the synthetic rate of GBM constituents is necessary in order to explain the increase in GBM mass which accompanies acute glomerular hypertrophy. At this early stage, alterations in the rate of degradation of membrane components play a minor role, if any; even a total inhibition of degradation, which itself is a very slow process [11], would not lead to the observed changes. In considering the subsequent phases of diabetic nephropathy, this review will later present evidence that, in addition to increased synthesis, decreased breakdown of basement membrane (BM) components, particularly the collagens, may participate in the accumulation of extracellular matrix.

Among the metabolic derangements that accompany the diabetic state, hyperglycemia represents a necessary, if not sufficient, factor for the development of some of the renal manifestations of diabetes mellitus. To examine the isolated influence of elevated ambient glucose on renal cell metabolism, and to avoid the complicating roles of an altered metabolic milieu or the effects of renal hemodynamics on the process of diabetic renal hypertrophy, we have previously developed a cell culture system in mouse proximal tubule epithelial cells where we could test the effects of elevated glucose concentration on cell growth and extracellular matrix biosynthesis [12]. Exposing the cells for 72 hours to serum-free medium containing 25 mM D-glucose resulted in significant cellular hypertrophy, as defined by an increase in cell size by cytofluorometry, and by increased total protein content and synthesis when compared to cells grown in 5.5 mM D-glucose [12]. This response was independent of changes in medium osmolality. Parallel studies also demonstrated that the high glucose medium stimulated the secretion of collagen type IV by approximately twofold. There was also a concordant increase in steady state levels of α1(IV) mRNA, providing evidence for increased collagen synthesis. Rates of collagen degradation were not measured in these short-term studies [12].

In streptozotocin-induced diabetic renal hypertrophy, in situ hybridization studies have demonstrated an early increase in collagen type IV mRNA in the hypertrophied proximal tubule cells [13]. Moreover, increased synthesis of collagen type IV is also a feature of other models of renal hypertrophy such as in compensatory hypertrophy following partial kidney ablation [14] and in angiotensin II-induced proximal tubule cell hypertrophy [15]. In the latter in vitro model, we have demonstrated that the hypertrophogenic effect of angiotensin II was additive to that produced by elevated ambient glucose [16].

In summary, the development phase of renal tubular hypertrophy in diabetes mellitus is accompanied by an early increase in the mass of the normal constituents of the TBM. In vitro studies provide evidence that high glucose levels, independent of any hormonal factors, appear to be a major stimulus for the increased synthesis of collagen type IV by proximal tubule cells and for the induction of hypertrophy of these cells.

TBM morphology in diabetes

The renal TBM and GBM, like some basement membranes in other tissues, undergo a progressive thickening which develops slowly over several years [17]. The increase in width of the TBM is almost uniform although splitting and layered thickening have also been described [18]. Views regarding TBM morphology in diabetes have long been based on subjective interpretation of biopsy or autopsy material, and in sharp contrast to the extensive studies on the GBM, there are virtually no detailed morphometric or ultrastructural studies devoted to the TBM in diabetes. Still lacking are large population surveys to derive data on mean and range of TBM width in normal adults similar to those involving the GBM [19]. Limited quantitative data were obtained in a study of renal biopsies from seven pairs of identical twins who were discordant for insulin-dependent (type 1) diabetes [20]. In each pair, the diabetic twin had a thicker TBM than the respective non-diabetic sibling. On average, the TBM of patients was 1100 nm, representing an almost 50% increase in thickness [20]. When it was possible to differentiate between proximal and distal tubules, there were no consistent regional differences between the widths of their basement membranes. Similarly, GBM width in the diabetic twins (average width approximately 500 nm) exceeded that in the nondiabetic twins in each instance (approximately 330 nm). In this study [20], values for muscle capillary basement membrane width in the diabetic twins did not differ from those in their siblings. These results were interpreted to indicate that the thickening of the TBM (and GBM) is a consequence of the metabolic perturbations of the diabetic state, rather than a generalized, hereditary disturbance in all basement membranes. Because only two of the diabetic cases had advanced glomerular lesions (characterized by hypertension, albuminuria and reduced creatinine clearance), it appears that TBM thickening occurs relatively early, that is, during the clinically-silent course of the disease.

Several studies utilizing immunohistochemical techniques have revealed conspicuous linear binding of albumin and IgG along the length of the thickened TBM in kidneys from diabetic subjects [21, 22]. It is generally accepted that this phenomenon represents passive entrapment of circulating proteins rather than an active immune process. It also remains speculative whether this abnormality plays any role in the development of TBM thickening.

TBM composition in diabetes

Limited available data suggest that, in addition to the generalized increase in TBM mass in diabetes (particularly the collagenous component), distinctive qualitative changes in the biochemical composition of the TBM also occur. Analysis of amino acid and carbohydrate content in post-mortem specimens from five diabetic subjects showed a significant increase in hydroxylysine (and a reciprocal decrease in lysine) in their TBM as compared with specimens from eight non-diabetic subjects [23]. This finding was similar to that reported for diabetic GBM in some, but not all studies [reviewed in 24–27]. There was also a relative increase in methionine and a relative decrease in half-cystine, valine, leucine and histidine [23]. Hydroxyproline content was only marginally increased, while the content of glycine, by far the most abundant amino acid residue, was not different [23].

The carbohydrate composition of the TBM in diabetic subjects was generally increased, due to significant increases in galactose and N-acetylglucosamine content, and only a slight increase in glucose content [23]. It should be noted that while this study [23] did not detect significant changes in sialic acid

residues in either TBM or GBM, multiple other studies have documented a significant reduction in renal sialic acid content [reviewed in 24–27]; this is possibly due to the increased activity of the degradative enzyme, sialidase, in the renal cortex [28]. Furthermore, significant reductions in sialic acid residues in renal matrices may not become evident until the diabetes is far advanced.

The macromolecular composition of the TBM in diabetes has been examined by immunohistopathologic studies. Using this technique, Falk et al [29] reported that the TBM of diabetic subjects undergoes polyantigenic expansion of various intrinsic basement membrane constituents including collagen types IV and V, laminin and fibronectin. These findings were encountered in early, moderate and advanced stages of diabetic nephropathy.

In investigations which focused on the monomeric elements which make up the ultrastructural assembly of collagen type IV molecules, Desjardins et al [30] utilized the protein A-gold immunocytochemical technique in kidney specimens from streptozotocin-diabetic rats and found that the labeling intensities of α1(IV), α2(IV), and α3(IV), but not α4(IV), were markedly increased along the TBM of diabetic kidneys. The fate of the recently discovered α5(IV) collagen chain in the diabetic TBM remains a subject of future investigation. However, it should be noted that in the normal kidney the localization of α5(IV) chain is virtually restricted to the glomerulus [31]. Nevertheless, it is evident from previous studies that the diabetic state may lead to disproportionate increases in the individual collagenous elements which comprise the renal basement membranes [29, 30], and may thus alter the functional properties of these structures.

Superimposed on the modifications in the intrinsic composition of the collagenous elements of renal matrices, the process of non-enzymatic glycation [32] can also impart further structural alterations which may play a role in further modifications in the metabolism and behavior of the matrix (see also below). It has been demonstrated that collagen type IV derived from the kidneys of diabetic subjects contains increased amounts of ketoamine-linked hexose, resulting from the non-enzymatic condensation of glucose with lysyl- and hydroxylysyl residues [33]. The TBM involvement by this process has been demonstrated in immunohistochemical studies in the kidney of diabetic rats [34]. In these studies, antiserum against glucitol-lysine localized the glycated protein to the TBM and the brush border of the proximal convoluted tubule; in control rats, only the brush border was weakly reactive. It can also be assumed that the long-lived TBM macromolecular constituents are further modified by the irreversible binding to advanced glycosylation end-products (AGE) [35, 36].

Increased collagen synthesis in diabetes

Several lines of evidence suggest that the increased content of extracellular matrix in the diabetic kidney is partly due to increased rates of synthesis, particularly in the early phases of the disease [27]. To some degree, this may relate to the kidney-specific anabolic effects of diabetes which stimulate the synthesis of RNA, proteins and structural glycoproteins [37, 38], and which represent crucial effector mechanisms for the development of renal hypertrophy [reviewed in 6, 39]. As discussed above, the fast accumulation of basement membrane material is commensurate with the induction phase of diabetic renal growth [10].

The study of the metabolism of renal basement membranes in diabetes has been virtually confined to isolated glomeruli or GBM [26, 27]. Conclusions regarding the status of TBM metabolism can only be indirectly implied from studies on whole kidney specimens. This may be a good approximation given the fact that the renal tubule comprises the bulk of the renal cortex.

Steady state levels of mRNA encoding extracellular matrix molecules have recently been measured in kidneys of animals with experimental diabetes in order to examine the mechanisms of increased matrix synthesis. Poulsom et al [40] found a twofold increase in laminin B1 mRNA in rat kidneys after 28 weeks following streptozotocin-induced diabetes. In contrast, levels for α1(IV) mRNA were not increased, and in fact showed a progressive decline with age in both the diabetic and the non-diabetic animals [40]. The study of Ledbetter et al in the obese KKAy mouse, a model of spontaneous non-insulin-dependent diabetes, reached opposite conclusions [41]. Renal cortical levels of α1(IV) mRNA were increased in the diabetic mice at four and six months of age (2- and 4-fold, respectively), while mRNA levels encoding laminin B1 and proteoglycan core protein were not different from those of age-matched non-diabetic mice. The level for α2(IV) mRNA was also increased in the kidneys of diabetic animals. The increase in collagen type IV mRNA in diabetic kidneys has been also reported in streptozotocin-diabetic mice [42]. Furthermore, in situ hybridization studies in streptozotocin-diabetic rats have localized the increase in α1(IV) mRNA to the straight segment of proximal tubule cells [13], implying increased TBM collagen synthesis. Renal cortical levels of fibronectin mRNA have also been shown to be variably increased in streptozotocin-induced diabetic rats (a 3-fold average increase) [43]. Taken together, these studies provide evidence that the diabetic state stimulates the synthesis of several extracellular matrix molecules in the kidneys of experimental diabetes, and that this effect is partly related to increased mRNA levels encoding these molecules. Hyperglycemia *per se* could represent a dominant factor in mediating this effect since high ambient glucose concentration in culture medium has been shown to increase the mRNA levels of several matrix moieties in glomerular mesangial cells [44] and α1(IV) mRNA levels in proximal tubule cells [12], as demonstrated in Figure 1.

Using nuclear run-off assays we demonstrated in the latter study [12] that collagen type IV gene transcription rate is stimulated when the proximal tubule cells are cultured in 25 mM D-glucose compared with 5.5 mM D-glucose. Transcriptional control of collagen type IV gene by high glucose media was also evident in experiments using transient expression studies (unpublished data). This involved the transfection of proximal tubule cells [12] with plasmid p184 [15] containing the CAT reporter gene linked to a genomic fraction of the mouse collagen type IV gene which includes the promoter and enhancer elements (gift of Dr. P. Killen). The results show (Fig. 2) that high medium glucose stimulates the CAT activity several-fold, thus providing evidence that high glucose modulates the interaction of putative transcription factors with specific *cis*-acting DNA sequences which regulate transcription.

There is also a general tendency towards increased specific activity of synthesis-related post-translational enzymes in the

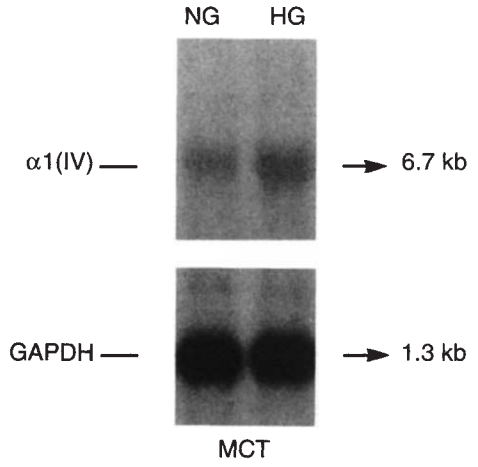

Fig. 1. *Autoradiogram of a northern blot in mouse cortical tubule (MCT) cells derived from proximal tubule.* Cells cultured for 48 hours in serum-free medium containing 450 mg/dl, or 25 mM glucose (HG) expressed more than twofold higher levels of the mRNA for the alpha-1 chain of type IV collagen than in 100 mg/dl, or 5.5 mM glucose (NG). The same filter was stripped and reprobed with GAPDH to control for equal RNA loading and transfer.

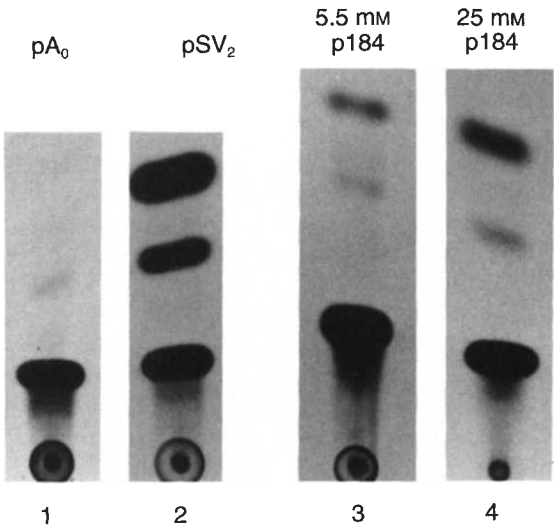

Fig. 2. *Transcriptional control of collagen type IV gene by elevated ambient glucose in cultured MCT cells, a mouse proximal tubule cell line.* The cells were transfected with various plasmids containing the chloramphenicol acetyltransferase (CAT) reporter gene, and CAT enzyme activity was then determined as previously described [12]. The autoradiographs show TLC-separated acetylated (upper bands), that is, CAT activity, from unacetylated ^{14}C-chloramphenicol. Lane 1: pAo-CAT, a negative-control promoterless plasmid; lane 2: pSV_2-CAT, a positive-control plasmid containing the SV40 promoter and enhancer; lane 3: p184, a plasmid containing a genomic fraction of the mouse collagen type IV gene which includes the promoter and enhancer elements (the MCT cells were cultured for 48 hours in 5.5 m D-glucose); and lane 4: MCT cells transfected with p184 but cultured for 48 hours in 25 mM D-glucose. The measured CAT activity in high-glucose culture was more than threefold higher than for normal-glucose culture (scintillation counting of cut-out bands). Not shown is the absence of any effect of ambient glucose on the CAT activity of the negative or positive control plasmids [12].

renal cortex of diabetic animals which can lead to increased collagen synthesis in the TBM and GBM [reviewed in 26, 27]. Many investigations have demonstrated, at least during some stages of the disease, increased activity of glucosyltransferase, galactosyltransferase, prolyl-4-hydroxylase and lysyl-hydroxylase [45, 46]. Additional evidence, although indirect, for enhanced collagen type IV synthesis in diabetes, has been derived from the demonstration of increased circulating levels of the disulfide-rich 7S domain of collagen IV, which also correlated with increased GBM synthesis [47] and the advanced degree of renal involvement by the diabetic state [48].

Decreased collagen degradation in diabetes

Little is known about the turnover rate of the TBM, however, it can be assumed to be quite slow if one considers what is known about the very slow turnover rate of other matrices such as the GBM, which can be quantitated over a period of several weeks [11, 49]. The degradative rate of the GBM is diminished further in streptozotocin-diabetic rats [50, 51].

The activities of several renal cortical enzymes involved in collagen degradation are diminished in experimental diabetes including α-glucohydrolase, β-D-galactosidase, and poorly characterized collagenase and lysosomal enzyme [52, reviewed in 26, 27].

It should be pointed out that increased collagen synthesis and decreased degradation are not mutually exclusive events and may be operating simultaneously but at different rates; increased synthesis may predominate early, while decreased degradation occurs later in the course of the disease [27].

Cellular mechanisms

In considering the effects of diabetes on the morphology and the metabolism of the TBM, we are faced with a scarcity of data regarding the cellular mechanisms which underlie these effects. In general, however, a few theories can be advanced to explain some of the pathophysiologic consequences of hyperglycemia, the predominant feature of the diabetic state.

Nonenzymatic glycation reactions resulting in both the early ketoamine linkages and the later-developing advanced glycosylation end-products (AGEs) often affect long-lived matrix constituents, particularly collagens, and may contribute in a significant way to aberrations in the structure and function of basement membranes [32, 35, 36, 53, 54]. Decreased degradation of matrix molecules may, in part, be a consequence of these reactions [55]. Future studies are required to elucidate the exact role of these abnormalities in the pathogenesis of diabetic nephropathy.

Increased flux through insulin-independent pathways for intracellular glucose utilization, particularly the polyol pathway, has been advanced as a contributory factor in mediating the early functional changes in certain diabetic target organs [56–58]. The resultant cellular dysfunction may relate to abnormalities in hormone responsiveness and signal-transduction pathways, which in part are induced by disordered cellular *myo*-inositol metabolism; *myo*-inositol supplementation may correct some of the disturbances that are attributed to increased activity of the polyol pathway [57, 58].

Evidence in favor of an involvement of the polyol pathway in some of the renal manifestations of diabetes has been provided

by our recent demonstration that the early glomerular hyperfiltration in streptozotocin-induced diabetic rats can be ameliorated by a diet supplemented with *myo*-inositol (1%), or by the administration of sorbinil, an aldose reductase inhibitor (ARI) which blocks the rate-limiting step in the polyol pathway (the conversion of glucose to sorbitol) [59]. These effects were not associated with changes in blood glucose levels or blood pressure, and they were specific in that the hyperfiltration of high protein feeding was not modulated by these maneuvers. The hyperfiltration in diabetic patients has also been favorably decreased by sorbinil therapy [60].

It is less clear whether increased activity of the polyol pathway is crucial in the genesis of the altered metabolism of renal extracellular matrices. Cohen, Klepser and Wu [61] could not reverse the under-sulfation of GBM proteoglycan by ARI administration to diabetic rats. Moreover, treatment with Statil, another ARI, did not reduce the increased levels of laminin B1 mRNA in rat kidneys [40]. However, a significant reduction in GBM thickness was demonstrated in diabetic rats after several months of sorbinil therapy [62]. Furthermore, a favorable response to reduce the microalbuminuria in diabetic rats has been reported with sorbinil treatment [63].

In our in vitro studies on the effects of elevated ambient glucose on proximal tubule cell growth and matrix production we found that the stimulation by high glucose of collagen type IV secretion and α1(IV) mRNA level was abolished when the cells were treated with 0.1 mM sorbinil [64] or when the high glucose medium was supplemented with 800 μM *myo*-inositol [65]. In contrast, the induction of cellular hypertrophy by high glucose was not modified by ARI treatment or *myo*-inositol treatment, suggesting that only particular actions of high glucose media are directly linked to the polyol pathway or to disturbances in *myo*-inositol metabolism.

An intriguing pathogenetic link between the products of the polyol pathway (and other pathways of glucose metabolism) and the reactions of non-enzymatic glycation has been proposed [66–68]. This is based on the observation that these glycation reactions can also involve metabolites of glucose which are the products of the polyol pathway (such as fructose) [66], and which can be further phosphorylated via novel pathways of glucose metabolism that are activated in diabetes mellitus [67, 68]. Sorbitol-3-phosphate, fructose-3-phosphate and other unidentified metabolites are increased in erythrocytes of diabetic subjects and could participate in protein glycation and cross linking [68].

Several of the manifestations of diabetic nephropathy may also be a consequence of altered production of local or circulating growth factors and/or modulations in the response to these factors [6, 9, 16, 69]. Analysis of the role of cytokines in the pathogenesis of diabetic changes is beyond the scope of this review. Transforming growth factor-β (TGF-β) deserves special consideration because it is produced by several cell types including renal cells, and it has a central role in promoting the synthesis and the accumulation of different extracellular matrix moieties [67, 70]. In recent studies [71], we gathered evidence that elevated ambient glucose levels in proximal tubule cell cultures increase TBF-β_1 mRNA levels and stimulate TBF-β bioactivity, which in turn may mediate some of the observed effects of high glucose levels on the cells. The importance of these observations to the pathogenesis of diabetic nephropathy will require further analysis.

Summary

TBM thickening and modifications in TBM composition in diabetes mellitus are primarily the consequence of the metabolic derangements of the disease. In particular, elevated ambient glucose is a major stimulus for the early induction of tubule cell hypertrophy and the accompanied increase in transcription and secretion of collagen type IV by these cells. In vitro studies indicate that increased activity of the polyol pathway, with the attendant alterations in cellular *myo*-inositol metabolism, may mediate the early increase in collagen type IV biosynthesis by proximal tubule cells. Some of the cellular effects of high glucose may also be mediated by bioactivation of local growth factors, such as TGF-β.

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