Rom J Morphol Embryol 2011, 52(1):75–79

ORIGINAL PAPER

RJME
Romanian Journal of Morphology & Embryology
http://www.rjme.ro/

Myofibroblast involvement in tubular basement membrane remodeling in type II diabetic nephropathy

E. MANDACHE[1,2)], MIHAELA GHERGHICEANU[2)], C. SERAFINCEANU[3)], M. PENESCU[1)], G. MIRCESCU[1)]

[1)] *"Carol Davila" Clinical Hospital of Nephrology, Bucharest*
[2)] *"Victor Babeş" National Institute of Pathology, Bucharest*
[3)] *"Nicolae Paulescu" Diabetes, Nutrition and Metabolic Diseases Institute, Bucharest*

Abstract

Diabetic nephropathy is always accompanied by tubulointerstitial damage. The mechanisms and the cells involved are not entirely clarified. The damaged tubules may regenerate or undergo necrosis or apoptosis. The purpose of this work was to investigate the structural transformations of both interstitial cells and extracellular matrix of the kidney stromal area in patients with type II diabetes mellitus associated with diabetic nephropathy. Tubulointerstitial fibrosis is characterized by loss of renal tubules and interstitial capillaries and the accumulation of extracellular matrix proteins. Tubular basement membranes were found to be a target of the remodeling process of the stromal area. Thickening, splitting and duplication were the main lesions of these membranes. Much attention has been focused on the importance of myofibroblasts in the progression of renal fibrosis. The results represent strong arguments for a direct involvement of myofibroblasts in the process of renal interstitial remodeling, tubular basement membrane thickening, and stromal fibrosis in the late stages of diabetic nephropathy.

Keywords: diabetic nephropathy, tubular basement membrane, myofibroblasts, fibronexus, alpha-smooth muscle actin.

Introduction

Diabetic nephropathy is a clinical syndrome in a patient with known or unknown diabetes mellitus characterized by persistent albuminuria, progressive proteinuria, hypertension and renal failure. One of the pathologic hallmarks of this disease is the progressive increase of extracellular matrix (ECM) in the renal parenchyma.

The earliest tubulointerstitial change in diabetic nephropathy is the thickening of tubular basement membrane (TBM), a similar phenomenon as the glomerular basement membrane (GBM) thickening. During this chronic nephropathy, tubules become progressively atrophic and the interstitium develops fibrosis and chronic inflammation. Except for this marked TBM thickening, the chronic tubulointerstitial changes resemble those seen with any other progressive glomerular disease [1].

It is already known that myofibroblasts are deeply involved in the synthetic activity of extracellular matrix components [2].

Myofibroblasts are cells occurring in stress conditions as primordial emergency cells, devoted to local wound healing, and contributing to tissue remodeling through its ability of synthesis and organization the ECM. This activity can severely impair the organ function when ECM proteins secretion becomes excessive in such cases as hypertrophic scars, scleroderma, Dupuytren's disease as well as in heart and kidney fibrosis [3].

Myofibroblasts have a spindle- or stellate-cell morphology surrounded by an abundant pericellular matrix. Their cytoplasm is palely eosinophilic and contains a prominent rough endoplasmic reticulum (rER) and a Golgi apparatus producing collagen secretion granules. These cells contain a peripheral layer of myofilaments and have no outer lamina. The myofilaments with focal densities are the same as those in smooth-muscle cells but are less numerous and found mostly just under the cell membrane. Bundles of myofilaments are also referred as stress-fibers. They correlate with immunoreactivity for α-SMA, the most commonly used molecular marker. Their immunophenotype is also positive for vimentin and fibronectin, but not for desmin [4].

Particular for myofibroblasts are their specific junctions called fibronexus. This is a cell-to-matrix junction and consists of two filament systems converging on points on the cell surface. A bundle of smooth muscle myofilaments attaches to the membrane, at a point marked by a plaque of dense proteins, and on the external surface of this point, the fibronectin filaments attach.

Materials and Methods

Kidney biopsies of 30 diabetic patients type II (48% males, 21.8% aged over 60 years, HBP present in 65.2%, and proteinuria was over 3.5 g/day in 70%) were obtained by percutaneous bioptic puncture using 16G guillotine needles. Each kidney sample has been divided in two parts: one fragment for immunofluorescence (IF), and a second group of 1-mm^3 small fragments for light microscopy (LM) and electron microscopy (EM).

For immunostaining samples were immediately snap frozen and then cryo-sectioned. The 4-μm thick cryo-sections were processed using ten FICT conjugated antibodies, including anti-α-SMA followed by DAPI.

For LM and EM fixation was performed with 4% buffered glutaraldehyde followed by 1% buffered osmic acid. After dehydration in ethanol, the 1-mm^3 fragments were embedded in Epon. One-micron thick sections stained with 1% Toluidine Blue were used for light microscopy and 70 nm thin sections, double stained with uranyl acetate and lead citrate, were performed for electron microscopy.

Diabetic nephropathy was diagnosed on morphologic ground (glomerular sclerosis, either nodular or diffuse), immunostaining criteria such as linear fluorescent deposits for anti-IgG and anti-albumin antibodies on the glomerular basement membranes (GBM), and thickened glomerular and tubular basement membranes in electron microscopy.

Results

After a full examination, all samples proved to be representative from the point of view of glomeruli number. The glomeruli were present between 7 and 20 for each patient. All samples showed signs of diabetic nephropathy labeled according to the Tervaert TW *et al.* [5] classification as stage II three patients, stage III 21 patients, and stage IV six patients. In brief, this four classes appointment of kidney pathologic features is taking into consideration the EM proven GBM thickening (class I), a mild (class IIa) or a severe mesangial expansion (class IIb), the occurrence of nodular sclerosis (Kimmelstiel–Wilson lesion) (class III), and the global glomerular sclerosis in over 50% of glomeruli (class IV).

Apart of the glomerular lesions, we have noticed in both light and electron microscopy a few tubules showing significant structural transformation concerning epithelial cells and TBM. Some had hypertrophic epithelial cells, some were atrophic, and some showed a much thickened, stratified or double layered tubular basement membrane (Figures 1 and 2). The interstitial space was enlarged and contained more collagen fibers, extracellular matrix (ECM) and interstitial cells, both fibroblasts and migratory cells like macrophages and lymphocytes. From place to place, the stromal aspect of some tubules showed the TBM in close contact with flattened interstitial cells. The ultrastructural investigation of these cells emphasized some characteristic features of myofibroblasts such as developed rough endoplasmic reticulum and Golgi apparatus, peripheral myofilaments, centrioles, peripheral dense plaques known as fibronexus and no external lamina on the cell surface. These cells were either spindle or stellate-shaped (Figure 3).

The ultrastructure of tubules with double-layered basement membranes showed the inner TBM layer in contact with the epithelial cells, being almost normal or slightly lamellated, while the outer layer was thicker and more or less sinuous. The outer aspect of these tubular basement membranes, in contact with the stromal area, also showed some close contacts with spindle typical fibroblastic and dendritic cells.

Apart of these stromal spindle cells facing the TBMs on the interstitial area aspect, we observed that the tubules having the TBM structured in two or three distinct layers showed cytoplasmic fragments or entire cell bodies clamped in between these layers. These apparently spindle-shaped cells, placed in between the TBM layers, were most probable large flattened cells still active or suffering apoptosis. From the ultrastructural point of view, they could be identified as myofibroblasts upon the specific cell-matrix junctions – fibronexus (Figures 4 and 5). Concerning the tubular epithelial cells, they were usually flattened and some of them showed basal bundles of filaments, oriented towards the TBM side (Figure 6).

In immunofluorescence, α-SMA positive elongated cells were found in the interstitial area, somehow placed around tubules (Figure 7) and glomeruli. Some of the nuclei visualized with DAPI were clearly embedded in α-SMA positive cytoplasm (Figure 8). Around some tubules, the α-SMA positive cytoplasm was structured in several layers (Figure 9).

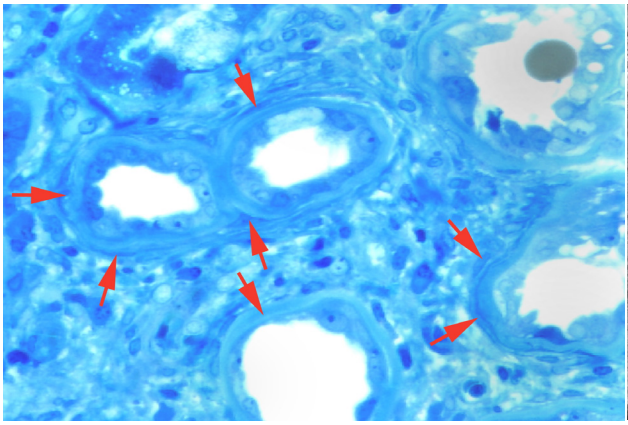

Figure 1 – ***Tubulointerstitial area of a diabetic nephropathy. Five cross-sectioned tubular profiles with thickened TBMs. In some zones the TBM is double layered and contain, or is surrounded by interstitial spindle cells (arrows). LM. Toluidine Blue, ob. ×100.***

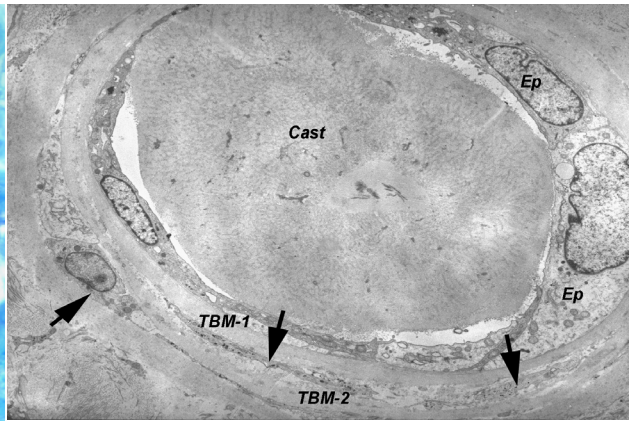

Figure 2 – ***Atrophic renal tubule showing flattened epithelial cells (Ep) and a protein cast. The original tubular basement membrane (TBM-1) is thickened and surrounded by a second, newly synthesized membrane (TBM-2). Several myofibroblasts can be seen (arrows) in between the tubular basement membranes. EM, low magnification.***

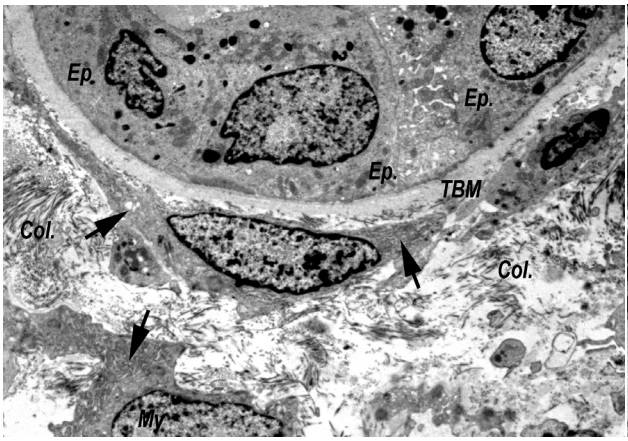

Figure 3 – ***Segment of a distal convoluted tubule showing epithelial cells (Ep.), tubular basement membrane (TBM), surrounding interstitial cells, and the extracellular matrix with many collagen fibers (Col.). The two myofibroblasts (My) are spindle- and stellate-shaped, and contain much developed rER and Golgi (arrows). EM, ×12 000.***

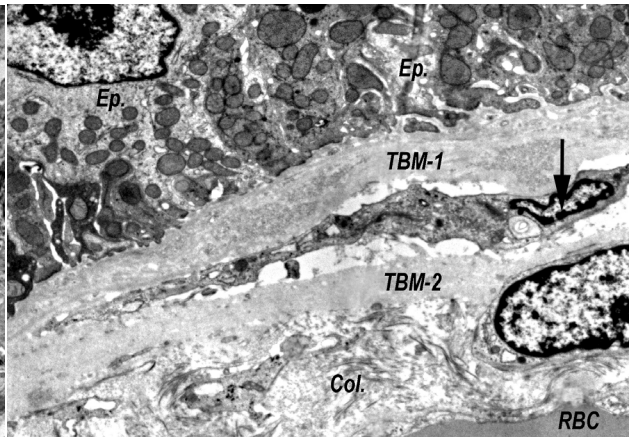

Figure 4 – ***Basal poles of tubular epithelial cells (Ep.) and the double-layered lamellated tubular basement membrane (TBM-1 and TBM-2). Interstitial collagen (Col.) and a capillary with red blood cell (RBC). EM, ×26 000.***

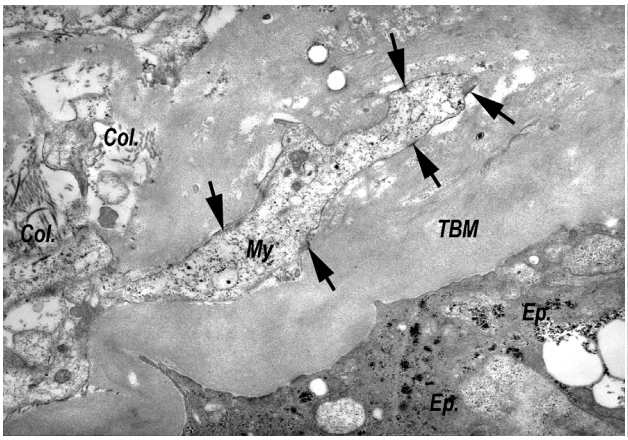

Figure 5 – ***Enlarged, double-layered tubular basement membrane (TBM) with a process of a myofibroblast in between (My). The cytoplasmic process shows free ribosomes, peripheral actin myofilaments and fibronexus elements (arrows). Epithelial cells (Ep.) and interstitial area with collagen type III fibers (Col.). EM, ×38 000.***

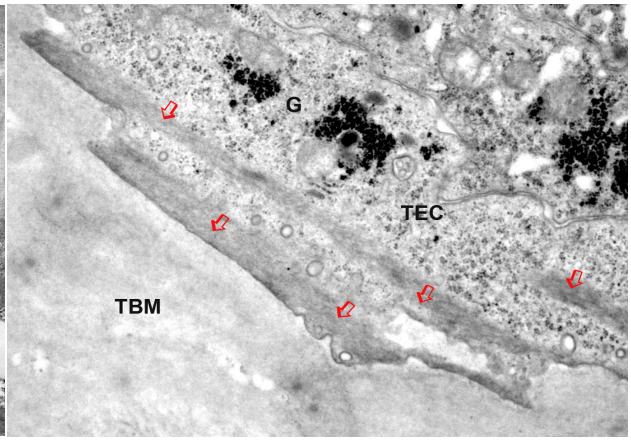

Figure 6 – ***The basal pole of an atrophic tubular epithelial cell (TEC). Actin filaments (arrows). Glycogen (G). Much thickened tubular basement membrane (TBM). EM, ×35 000.***

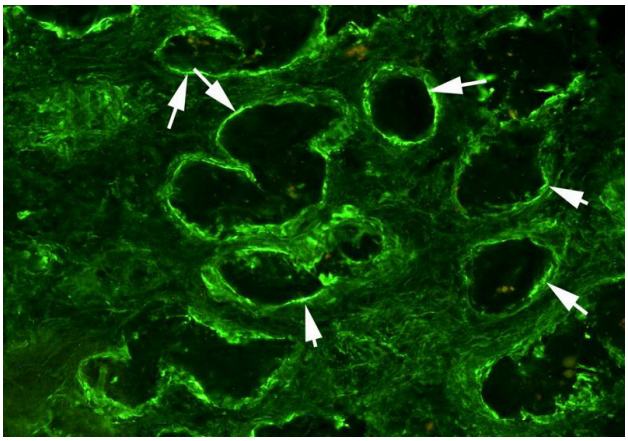

Figure 7 – ***Tubulointerstitial area on a tissue cryosection immunostained with anti-α-SMA, FITC conjugated antibody. The characteristic fluorescent marker for myofibroblasts is placed manly around tubules (arrows). IF, anti-α-SMA, ob. ×20.***

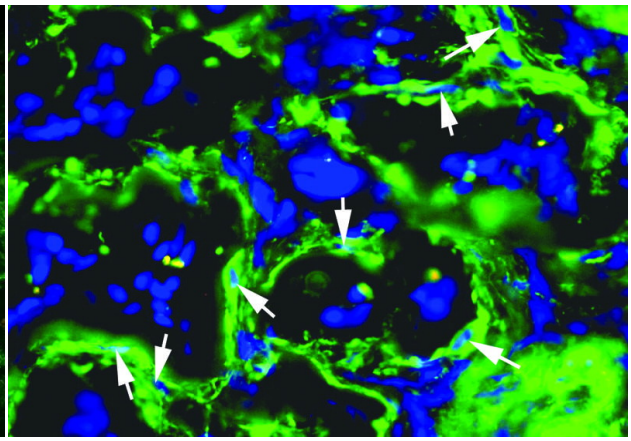

Figure 8 – ***Tubular profiles surrounded by myofibroblasts (white arrows). IF, anti-α-SMA and DAPI, ob. ×40.***

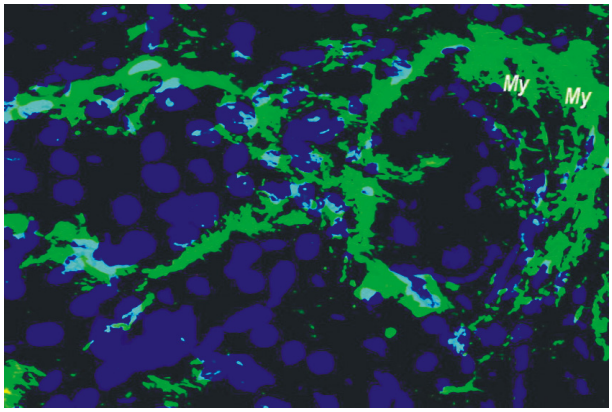

Figure 9 – ***Tubules with multilayered TBM and surrounding myofibroblasts (My). IF, anti-α-SMA and DAPI, ob. ×40.***

The position of α-SMA positive cells and of the corresponding spindle cells in EM was almost similar. Both the ultrastructure and the presence of cytoplasmic α-SMA, suggest that these cells were myofibroblasts. Thus, myofibroblasts occur in stress conditions and contribute to interstitial remodeling of the stromal main components.

Discussion

A constant structural feature of chronic renal failure is the tubulo-interstitial fibrosis. The stromal area of fibrotic kidneys contains both fibroblasts and myofibroblasts, beside some other migratory cells. The myofibroblasts, which are usually not present in the steady state of healthy people, are supposedly responsible for the increased synthesis of extracellular matrix [6]. Classic histological features of myofibroblasts disclose a plump -spindle-cell morphology with a pale, ill-defined cytoplasm compared with the more fibrilar smooth-muscle cells. Myofibroblasts are positive for α-smooth-muscle actin, vimentin, fibronectin and only sometimes desmin. From the ultrastructural point of view, most obvious are the rough endoplasmic reticulum and some myofilaments with focal densities known as stress fibers, and a specialized junctional structure [7].

The cells we have investigated, both in electron microscopy and in immunofluorescence, were suited well to this description. They were spindle shaped and had long cytoplasmic processes. Beside these, they showed the typical cell–matrix junctions, the fibronexus, which can be considered an electron microscopic marker of myofibroblasts. The positiveness for α-SMA in IF is corresponding to the presence of intracellular actin filaments. The fibronexus is in fact a contact structure between the myofibroblasts and the extracellular matrix that mediates the continuity of intracellular actin filaments with extracellular matrix proteins including fibronectin. These proteins constitute long fibronectin fibrils [8].

The location of α-SMA positive cells in immunofluorescence and of the corresponding spindle cells in EM was definitely similar. This location was always in the interstitial space, either around the TBM or in between its layers. Both EM and IF features of these cells suggest that they are myofibroblasts.

Thus, myofibroblasts are most probable involved in the synthesis of TBM and ECM specific proteins, leading to TBM thickening and duplication by generating a second and a third layer, and finally to renal tubulointerstitial fibrosis.

We did not find α-SMA positiveness inside the TBM, meaning in the tubular epithelial cells (TEC). This assessment seems to be in disagreement with the presence of basal filaments organized in bunches in some epithelial cells. The presence of these basal bundles of filaments, clearly seen in EM, is one of the strong arguments in favor of the epithelial-myofibroblast transformation theory [9]. The latter report mention the loss of epithelial cells polarity and junctions, followed by detachment from the TBM and neighboring cells, and their migration into the peritubular interstitium, through a damaged TBM. In disagreement with these authors, we could not find any TBM disruption or transfer of epithelial cells to the stromal space. Our opinion, in agreement with Ru Y *et al.* [10], is that the occurrence of stromal myofibroblasts in patients with diabetic nephropathy has an alternative origin, most probably through resident fibroblasts or bone marrow derived cells.

Myofibroblasts play an important role in the remodeling of tubulointerstitial area of diabetic nephropathy patients [11, 12]. This activity is most obvious in diabetic nephropathy of class III and IV. Their contribution is essential in the neo-formation of extracellular matrix, TBM thickening and duplication included. The TBM thickening is simultaneously inducing TEC ischemia and atrophy. Thus, the stromal fibrosis is closely followed by the end-stage nephropathy.

Conclusions

We strongly suggest the existence of a direct relation between the number of myofibroblasts and the TBM thickening and duplication and interstitial fibrosis. Although observed only in diabetic patients, the same process of stromal remodeling and interstitial fibrosis can be a common final way in many other chronic nephropathies with renal failure.

Acknowledgements

This work was supported by CNCSIS–UEFISCSU, Project No. 1182/2009–ID599 PN II – IDEI code/2008.

References

[1] Fogo AB, Bruijn JA, Cohen AH, Colvin RB, Jennette JC, *Fundamentals of renal pathology*, Springer-Verlag, 2006, 132.

[2] Hinz B, Phan SH, Thannickal VJ, Galli A, Bochaton-Piallat ML, Gabbiani G, *The myofibroblast: one function, multiple origins*, Am J Pathol, 2007, 170(6):1807–1816.

[3] Eyden BP, *The myofibroblast: a study of normal, reactive and neoplastic tissues, with an emphasis on ultrastructure. Part 2 – Tumours and tumour-like lesions*, J Submicrosc Cytol Pathol, 2005, 37(3–4):231–296.

[4] Eyden BP, *The myofibroblast, electron microscopy and cancer research*, Int J Cancer, 2009, 125(7):1743–1745; author reply 1746.

[5] Tervaert TW, Mooyaart AL, Amann K, Cohen AH, Cook HT, Drachenberg CB, Ferrario F, Fogo AB, Haas M, de Heer E, Joh K, Noël LH, Radhakrishnan J, Seshan SV, Bajema IM, Bruijn JA; Renal Pathology Society, *Pathologic classification of diabetic nephropathy*, J Am Soc Nephrol, 2010, 21(4):556–563.

[6] Picard N, Braum O, Vogetseder A, Kaissling B, Le Hir M, *Origin of renal myofibroblasts in the model of unilateral ureter obstruction in the rat*, Histochem Cell Biol, 2008, 130(1):141–155.
[7] Eyden BP, *Electron microscopy in the study of myofibroblastic lesions*, Semin Diagn Pathol, 2003, 20(1):13–24.
[8] Eyden BP, *Brief review of the fibronexus and its significance for myofibroblastic differentiation and tumor diagnosis*, Ultrastruct Pathol, 1993, 17(6):611–622.
[9] Ng YY, Huang TP, Yang WC, Chen ZP, Yang AH, Mu W, Nikolic-Paterson DJ, Atkins RC, Lan HY, *Tubular epithelial-myofibroblast transdifferentiation in progressive tubulo-interstitial fibrosis in 5/6 nephrectomized rats*, Kidney Int, 1998, 54(3):864–876.
[10] Ru Y, Eyden B, Curry A, McWilliam LJ, Coyne JD, *Actin filaments in human renal tubulo-interstitial fibrosis: significance for the concept of epithelial-myofibroblast transformation*, J Submicrosc Cytol Pathol, 2003, 35(3):221–233.
[11] Adler S, *Diabetic nephropathy: linking histology, cell biology, and genetics*, Kidney Int, 2004, 66(5):2095–2106.
[12] Essawy M, Soylemezoglu O, Muchaneta-Kubara EC, Shortland J, Brown CB, el Nahas AM, *Myofibroblasts and the progression of diabetic nephropathy*, Nephrol Dial Transplant, 1997, 12(1):43–50.

Corresponding author
Eugen Mandache MD, PhD, Department of Nephropathology, "Carol Davila" Clinical Hospital for Nephrology, 4 Griviței Avenue, Sector 1, 010731 Bucharest, Romania; Phone +40727–170 204, e-mail: mandache.eugen@yahoo.com

Received: October 10th, 2010

Accepted: January 24th, 2011