MORPHOLOGY OF RENAL INTERSTITIAL FIBROBLASTS

Kostadinova-Kunovska S¹, Jovanovic R¹, Janevska V¹, Grchevska L², Polenakovic M³, Petrushevska G¹

¹ Institute of Pathology, Faculty of Medicine, Ss. Cyril and Methodius University, Skopje, R. Macedonia
² Department of Nephrology, Clinical Centre, Skopje, R. Macedonia
³ Macedonian Academy of Sciences and Arts, Skopje, R. Macedonia

Abstract: Introduction: Renal fibrogenesis is a process common to all progressive kidney diseases. The main executive cell in this process is the fibroblast, by secreting and remodelling the extracellular matrix. The number of fibroblasts is minor in a healthy kidney interstitium, but it increases during the process of fibrosis. Their morphology and immunophenotype vary due to different intrinsic and extrinsic factors which makes their identification and visualization, as well as determination of their origin, very difficult.

Material and methods: We performed morphological and immunohistochemical analyses on kidney biopsies with interstitial fibrosis, using the following antibodies: Vimentin, α-SMA, S100A4, Cadherin 9 and CD34. We also did light-microscopy analyses of semi-thin sections of tissue embedded in epoxy resin and stained with Toluidine blue.

Results: Our observations show that different cells in the fibroblastic population show positivity for different markers, thus contributing to the theory that there are different subpopulations of fibroblasts, with different origins, that take part in renal fibrogenesis.

Key words: interstitial fibrosis, fibroblasts, morphology.

Introduction

The renal interstitium, defined as the extravascular intertubular space of the renal parenchyma, is bounded on all sides by tubular and vascular basement
membranes, and its volume varies between 7 and 9% in the cortical compartment and 30–40% in the inner medulla [1, 2].

The interstitium is not only a "passive space" providing support for the functional renal units, but it also mediates and modulates almost all exchange processes among tubular and vascular renal spaces, influences the glomerular filtration, and produces a variety of local and systemic hormones, such as erythropoietin [3], adenosine, prostaglandins, etc.

The interstitium is composed of cellular elements and an extracellular matrix (ECM), which is made of ground substance and fibrillar structures. The ground substance consists of proteoglycans, glycoproteins and interstitial liquid, and the fibrillar reticulum is made of fibres of collagen I, III, VI, as well as collagen types IV and V, which are found in the basement membranes, also considered as part of the interstitium.

Various types of interstitial cells are embedded in this environment. They fill the irregular spaces between tubules and vessels and their density increases with the increment of the total interstitial volume.

The presence of fibroblasts (or fibroblast-like cells), macrophages, dendritic cells and lipid-laden interstitial cells has been documented in different proportions in various interstitial compartments [1, 4].

Fibroblasts produce an extracellular matrix and are rarely appreciated in the normal renal interstitium. Their number increases during the fibrotic processes and they are considered to be: (1) resident and proliferate when stimulated by various mediators during renal tissue damage; (2) newly-formed, during the process of epithelial-mesenchymal transition (EMT), or, according to recently published data (3) form the bone marrow [5–9].

Objective

The objective of this paper is to visualize and analyse the immunophenotype of fibroblasts in kidneys with interstitial fibrosis.

Material

We used 30 renal biopsy samples, previously diagnosed as primary glomerulopathy by means of standard histological and immunofluorescent examination. Only biopsies containing at least 5 glomeruli, cortical tubulointerstitium and arteriolar segments were taken into consideration, and the main inclusion criterion was interstitial fibrosis of at least 10% in the renal cortex.
As controls, we used tissue samples from normal adult kidneys obtained for analysis for renal carcinoma which did not affect the surrounding renal parenchyma.

Methods

After obtaining the kidney biopsies and separating small fragments from both ends of the biopsy samples for electron microscopy, the rest of the kidney tissue, as well as the tissue samples from the nephrectomies used as controls, were fixed in 10% neutral buffered formaldehyde for a period of 18–24 hours, embedded in paraffin and cut into 4–5 µm sections. Tissue samples from all cases were initially stained with haematoxylin-eosin, PAS, trichrom – Masson and silvermethenamine – Jones. All these histochemical stainings had been used for diagnosis, and afterwards for selection of cases included in this study.

Immunohistochemical stainings on tissue sections from all biopsy and control cases were performed in a semi-automated manner with the EnVision FLEX visualization system (DAKO, Denmark). The tissue samples were pretreated in a PT Link from DAKO, Denmark, after which the staining procedure continued manually in a humid dark chamber. The following monoclonal and polyclonal antibodies were used for staining: Vimentin – clone V9 (DAKO), αSMA – clone 1A4 (DAKO), S100A4 – polyclonal (DAKO), Cadherin 9 – polyclonal (Atlas Antibodies) and CD34 – clone QBEnd-10 (DAKO).

For Vimentin, αSMA and CD34 we looked for an internal positive control, for S100A4 we used a lymph node as a positive control and for Cadherin 9 we used placental tissue as a positive control. The negative control stainings were done by omitting the application of the primary antibody to a separate the tissue section.

Furthermore, samples of the biopsies intended for electron microscopy were embedded in epoxy resin, cut into semi-thin sections (1 µm thick), stained with Toluidine blue and analysed on a light microscope.

We performed qualitative light-microscopic analyses with a NIKON Eclipse 1000 microscope and the pictures were taken with the Olympus Cell A image acquisition software.

Results

The analysed series consisted of biopsies from 18 male (60%) and 12 female (40%) patients at a mean age of 43.1 (min = 13, max = 82). The control group consisted of samples from 6 male (60%) and 4 female patients (40%) at a mean age of 52.6 (min = 29, max = 74).
The analysed cases had all been previously diagnosed with primary glomerulopathy (7 membranous glomerulopathy, 8 IgA nephropathy, 8 focal segmental glomerulosclerosis, 3 membranoproliferative, and 4 extracapillary glomerulonephritis).

The analyses showed interstitial fibrosis with focal rather than diffuse distribution in all analysed cases, which was not observed in the control cases. The presence of interstitial cells was markedly increased in the fibrotic foci in comparison to the non-fibrotic portions of the kidney biopsies, as well as to the controls.

The microscopic analyses showed that the majority of the interstitial cells were inflammatory cells, and the other, less prominent, population were spindle or stellate-shaped cells, with abundant branched or bipolar cytoplasm, an oval nucleus and prominent nucleolus/i, as seen on the semi-thin sections (Fig. 1). The latter had a predominantly concentric orientation around the atrophic tubules, or were haphazardly arranged in the fibrotic foci.

![Figure 1 – Semi-thin section stained with Toluidine blue (x 1000) showing interstitial fibroblast (arrow)](image)

On immunohistochemical analyses, all of these cells were positive for Vimentin (Fig. 2), and different proportions of them showed positive signals on the stainings with α-SMA (Fig. 3), S100A4 (Fig. 4) and Cadherin 9 (Fig. 5). We also observed that not always the same cells were positive for all markers. On the contrary, different cells showed positivity for different markers.

Besides for the above-mentioned, so-called fibroblast markers, we found a minority of cells in some fibrotic foci showing positivity for CD34 (Fig. 6).

Apart from that, a minor proportion of atrophic tubular epithelial cells in the fibrotic environment also showed positivity for Vimentin, α-SMA and S100A4.
Figure 2 – Vimentin positive fibroblasts and atrophic tubular epithelial cells in fibrotic area (x 200)

Figure 3 – α-SMA positive fibroblasts in interstitial fibrotic focus (x 200)

Figure 4 – S100A4 (FSP1) positive interstitial fibroblasts (arrow) and atrophic tubular epithelial cells (x 400)
Discussion

The deposition of an increased quantity of ECM in the renal interstitium, interstitial fibrosis, is a common denominator for all progressive renal diseases [10]. The pathogenetic mechanisms leading to interstitial fibrosis are considered to be the same in different types of renal injury. The destructive fibrosis in the tubulointerstitial renal compartment initiates a vicious circle that successively worsens the renal function.

The fibroblasts are executive cells in the process of interstitial fibrosis. It is considered that there are three subtypes of interstitial fibroblasts, depending on their origin: (i) resident fibroblasts; (ii) fibroblasts originating from epithelial cells during the process of epithelial-mesenchymal transition and (iii) fibroblasts originating from the bone marrow.
Different pathological processes create an environment of continual excessive production of numerous mediators, such as chemokines and growth factors, stimulating deposition of ECM by activating and increasing the number of interstitial fibroblasts [10–12].

The study of renal fibroblasts is difficult due to their paucity in healthy kidney tissue; their admixture with other interstitial cells, especially in an inflammatory environment, which makes them hardly visible; predominance of the surrounding tubular and vascular structures and collapse of the interstitial space due to fixation artefacts [13]; as well as a lack of specific markers for this cell population.

In our study, the fibroblasts in the fibrotic foci of the analysed cases, as well as the few ones identified in the controls, were positive for Vimentin. Vimentin is an antibody directed against the intermediate filaments of the mesenchymal cells, and therefore presents with cytoplasmic positivity in cells of mesenchymal origin.

However, a smaller proportion of them in the fibrotic areas of the diseased kidney specimens were positive for α-SMA, unlike the control cases, where they were not found. αSMA is marking the actin cytoskeleton of the myocytes and myofibroblasts, giving a cytoplasmic signal.

The positivity for smooth muscle actin renders these cells as "myofibroblasts", cells with contractile properties [8, 14–16], which are considered to be activated fibroblasts and, therefore, responsible for the deposition of ECM. However, the significance of α-smooth muscle actin (α-SMA) expression, which is the major morphological characteristic of myofibroblasts, remains to be determined in detail.

Strutz et al. [17] suggested that S100A4 (also known as FSP1 – fibroblast specific protein) marks fibroblasts both in healthy and in fibrotic kidneys. S100A4 is a member of the S100 family of calcium-binding proteins, which have been associated with various cellular events, including growth, signalling, differentiation and motility. It has been widely used as a fibroblast marker due to its putative role in cell shape changes, particularly in cells induced to elongate [18], where it gives both cytoplasmic and nuclear positivity.

Studies have shown an increased number of S100A4 positive cells in foci of interstitial fibrosis [19, 20], as was verified in our series, too.

The presence of positive atrophic tubular epithelial cells, also noted in our series, indicated FSP1, besides Vimentin and αSMA, as a marker for identification of epithelial cells undergoing mesenchymal transformation during fibrogenesis [7, 8], the so-called epithelial-mesenchymal transition (EMT). This phenomenon has been a matter of extensive investigations during recent years, resulting in vibrant discussions about its existence in human tissue in vivo.
While it is considered by some research groups to be a major source of fibroblasts (about 36% of new fibroblasts) that excessively produce ECM [7, 8], others claim that pericytes are the precursor cells for myofibroblasts [21–23].

Other investigations have shown that not only interstitial fibroblasts, but also a subset of mononuclear inflammatory cells expresses FSP1 [13, 24–27]. We also had a positive nuclear and cytoplasmic signal in the surrounding mononuclear inflammatory cells, which hampered the visual identification of the fibroblasts, thus making FSP1 an unreliable marker for fibroblasts.

Recently, Cadherin 9 has been proposed to be a more specific marker for interstitial fibroblasts [28]. Cadherin 9 is a member of the Cadherin superfamily of calcium-dependent cell adhesion proteins. It mediates homophilic cell-cell interactions, and is also thought to be involved in signal transducing, thus manifesting cytoplasmic and/or membranous positivity.

Thedieck et al. [28] have shown that Cadherin 9 positive cells were present only among interstitial cells, and it has not been detected in the epithelial, endothelial or glomerular cells. These observations were common for both normal and fibrotic renal tissue, with marked abundance of positive cells in the latter. They also supported these observations with analyses on renal fibroblast cell lines, where only fibroblasts from fibrotic kidneys, and not from normal kidneys, were positive for Cadherin 9.

In our series, Cadherin 9 positive interstitial cells were present in the fibrotic areas, yet in much smaller number than those positive for other previously mentioned markers, which may indicate that Cadherin 9 positive fibroblasts are a subset of the renal interstitial fibroblast population.

In support of the third theory for renal fibroblast origin, we found a subset of fibroblasts positive for CD34, which is considered to be a marker for progenitor hematopoietic cells and for endothelial cells and has originally been discovered on the surface of bone marrow stem cells. The bone marrow has been considered to be a source for fibroblasts during fibrogenesis by many authors [29–31], but their existence needs further elucidation.

The above-presented results and literature data confirm the statement that fibroblasts are not a homogenous cell population; they express different proteins in various functional stages, which makes their identification and characterization very difficult and subtle.

Acknowledgement

We wish to thank Prof. Claudia Mueller from the Medical University Clinic and Center for Medical Research in Tuebingen, Germany for providing the antibody for Cadherin 9.
REFERENCES


Резиме

МОРФОЛОГИЈА НА БУБРЕЖНИТЕ ИНТЕРСТИЦИЈАЛНИ ФИБРОБЛАСТИ

Костадинова-Куновска С.1, Јовановиќ Р.1, Јаневска В.1, Грчевска Л.2, Поленаковиќ М.3, Петрушевска Г.1

1 Институт за биолошки и медицински факултети, Скопје, Р. Македонија
2 Клиника за нефрологија, Клинички центар, Скопје, Р. Македонија
3 Македонска академија на науките и уметностите, Скопје, Р. Македонија

Ввод: Фиброгенезата е процес кој се случува кај сите прогресивни бубрежни заболувања. Главната извршна клетка во овој процес е фибробластот, кој продуцира и го ремоделира екстрацелуларниот матрикс. Во нормалниот бубрежен интерстициум има мал број фибробласти, но нивниот број се зголемува во текот на процесот на фиброза. Нивната морфологија и имунофенотип се разликуваат под влијание на различни внатрешни и надворешни фактори, што ги отежнува нивната идентифицира, визуелизација и одредувањето на нивното потекло.

Материјал и методи: Направивме морфолошки и имунохистохемиски анализи на бубрежни биопсии со интерстицијална фиброза, со примена на следниве антитела: Vimentin, α-SMA, S100A4, Cadherin 9 и CD34. Направивме, исто така, светлосно-микроскопска анализа на полутенки пресечи на ткиво вкалапено во смола и боено со Toluidine blue.

Резултати: Нашите анализи покажаа дека различни клетки во фибробластната популација покажуваат позитивитет на различни маркери, што едни во прилог на теоријата дека во бубрежната фиброгенеза учествуваат различни субпопулации на фибробласти со различно потекло.

Ключни зборови: интерстицијална фиброза, фибробласти, морфологија.

Corresponding Author:

Kostadinova-Kunovska S
Institute of Pathology, Faculty of Medicine
Ss. Cyril and Methodius University
50 Divizija 6b
1000 Skopje, R. Macedonia
Tel.: +38923104687
E-mail: skkunovska@yahoo.com