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Original article

Epithelial-mesenchymal transition in pediatric nephropathies

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ABSTRACT

Introduction: Epithelial-mesenchymal transition (EMT) is a process in which epithelial cells may express mesenchymal cell markers with subsequent change in their functions, and it may be part of the etiopathogenesis of kidney disease.
Objective: The aim of this study was to evaluate the immunexpression of some EMT inducers and markers in frequent nephropathies in pediatric patients.
Methods: 59 patients aged 2–18 years old were selected and divided into 6 groups of frequent nephropathies in children and adolescents, as well as one control group. Urea and creatinine data of the patients were recorded. TGF-β3, fibronectin, α-SMA and vimentin were evaluated by immunohistochemistry.
Results: Glomerular TGF-β3 was higher in the Lupus Nephritis and Acute Diffuse Glomerulonephritis (ADGN) groups than in the control group. Glomerular fibronectin was higher in the Podocytopathy, Lupus Nephritis, ADGN and Membranous Glomerulopathy patients than in control subjects. The expression of α-SMA was higher in the tubulointerstitial compartment of ADGN and Membranous Glomerulopathy groups than in the control group. Glomerular α-SMA was higher in ADGN patients than in control and Berger's Disease groups. Glomerular vimentin was higher in individuals with ADGN than in those with Podocytopathy, Lupus Nephritis, Berger's Disease and Thin Basement Membrane Disease/Alport Syndrome. There was a positive correlation between fibronectin in the tubulointerstitial compartment and creatinine levels, between α-SMA and vimentin in both tubulointerstitial and glomerular compartments, and between urea and creatinine levels of patients, regardless of their nephropathy ($p < 0.05$ for all results).
Conclusion: These markers may possibly be used as indicators of renal functional impairment in various nephropathies in pediatric patients.

1. Introduction

Epithelial-Mesenchymal Transition (EMT) is a process in which epithelial cells can change their morphology and phenotype, and express mesenchymal cell markers with subsequent change in their functions, such as acquisition of migratory capacity and interstitial matrix synthesis [1]. EMT may occur in the following situations: during embryogenesis, during tissue repair and fibrosis, and during the progression of cancer or methastasis [2].

Protein-coding genes that are responsible for cell adhesion, such as E-cadherin, desmoplakin and Zonula occludens-1 (ZO-1), or which are part of a group of intermediate filaments of epithelial cells, such as cytokeratin, are inhibited during EMT. On the other hand, cytoskeletal protein coding genes, such as vimentin and Alpha Smooth Muscle Actin (α-SMA), which are rearranged to allow for the translocation into the stroma, or proteins in the extracellular matrix itself, such as fibronectin, are stimulated [3].

TGF-β1 is one of the most studied and well-known inducers of EMT. This isoform can mediate EMT through different intracellular signalling pathways, and it is the main inducer of EMT involving renal fibrosis [4]. However, it was observed that TGF-β1 and TGF-

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β2 may be dependent on TGF-β3 during EMT induction, showing that the various TGF-β isoforms play essential roles in this process [5].

Regardless of the primary cause of interstitial fibrosis, its development is characterized by the presence of α-SMA positive activated fibroblasts, also called myofibroblasts. EMT is regarded as one of the hypothesis on the origin of these cells [6]. Progressive lesions and chronic kidney disease usually tend to cause fibrosis and subsequent loss of parenchymal function, which characterizes terminal kidney [3]. As EMT may be part of the etiopathogenesis of kidney disease, this biological process has been widely studied in nephropathies [7].

In a study by the Division of Nephropathology of the Federal University of Triângulo Mineiro (UFTM), the following glomerulopathies were found to be highly prevalent in children and adolescents: Podocytopathy (Focal Segmental Glomerulosclerosis—FSGS/Minimal Change Disease), Lupus Nephritis, Berger's Disease, Thin Basement Membrane Disease/Alport Syndrome, Acute Diffuse Glomerulonephritis (ADGN), and Membranous Glomerulopathy [8]. There are few studies on histopathological changes in renal biopsies of pediatric patients, as well as on the use of these descriptions as prognostic factors [9]. Because of this, the present study aimed to evaluate whether the immunexpression of some EMT inducers and markers such as TGF-β, fibronectin, α-SMA and vimentin can change depending on the clinical and morphological characteristics, as well as on the severity of frequent nephropathies in children and adolescents in the Division of Nephropathology of this institution, thus facilitating diagnosis and prognosis of these patients.

2. Materials and methods

This study was approved by the Research Ethics Committee of the Federal University of Triângulo Mineiro. Once the biopsies were requested, information regarding laboratory data (proteinuria, urea and creatinine) and epidemiological data (age, gender and ethnicity) of the patients was recorded.

Patients aged 2–18 years, who underwent renal biopsy from 1996 to 2010, were included in the study. A total of 48 subjects were selected and divided into 6 groups according to the frequent nephropathies in the pediatric age group [8]: Podocytopathy (FSGS/Minimal Change Disease) (N = 10), Lupus Nephritis (N = 10), Berger's Disease (N = 10), Thin Basement Membrane Disease/Alport Syndrome (N = 5), Acute Diffuse Glomerulonephritis (N = 8), and Membranous Glomerulopathy (N = 5). The data of the control group (N = 11) came from autopsies performed in 3–18 year-old individuals that did not have renal changes. All the groups were matched according to age.

In order to evaluate the amount of TGF-β1, TGF-β3, fibronectin, α-SMA and vimentin, the following primary antibodies were used: Anti- TGF-β1 (R e D Systems®), Anti-TGF-β3 (Abcam®), Anti-Fibonectin (Abcam®), Anti-Human Smooth Muscle Actin (α-SMA) (Dako®), and Anti-Vimentin (Dako®), diluted 1:40, 1:20, 1:120, 1:80 and 1:60, respectively. Positive and negative controls were selected according to the instructions of each antibody. Sections of prostate carcinoma, placenta, kidney, salivary gland and esophagus were used for the expression of TGF-β3, fibronectin, α-SMA, and vimentin, respectively.

During antigen retrieval, citrate buffer was used for α-SMA and vimentin, and EDTA buffer was used for TGF-β1, TGF-β3 and fibronectin at 97 °C. All antibodies were incubated for two hours at room temperature. Then LSAB+ System-HRP (DAKO®) was used in the developing process, and Diaminobenzidine (DAB) was used as a chromogen substrate.

The immunostained areas had a brownish color due to the precipitation of the chromogenic substrate (DAB), so this coloration was selected by the observer using Leica QWin Plus® interactive image analysis software. This software automatically identifies all regions which are stained with the same brownish coloration and expresses the result in percentage of stained area, regardless of cell type, including cells and extracellular matrix. The staining was quantified in two renal compartments: in the tubulointerstitial compartment, which was analyzed with a 40× objective (1600× total magnification), and in the glomerular compartment, which was analyzed with a 100× objective (3200× total magnification) in immersion oil. Vimentin and α-SMA antibodies showed a cytoplasmic and membranous staining pattern, whereas TGF-β1, TGF-β3 and fibronectin showed a cytoplasmic and extracellular staining pattern.

Sirius Red staining was performed in order to evidence collagen fibers, and the slides were examined under polarized light so as to quantify fibrosis. The collagen area in the scanned image, usually showing reddish birefringence, had been stained in order to obtain the percentage of fibrotic area per field, also by using Leica QWin Plus® interactive image analysis software only in the tubulointerstitial compartment.

Statistical analysis was performed using GraphPad Prism 5.00 software. In variables with normal distribution and similar variances, Anova test (F) was used followed by Tukey's test. In this case, the results were expressed as mean ± standard deviation. Otherwise, Kruskal-Wallis test (H) was used followed by Dunn's test. In this situation, the results were expressed as median (min – max). The correlation between two variables with normal and non-normal distribution was analyzed using Pearson (r) and Spearman (rS) tests, respectively. The differences in which the p value was less than 5% ($p < 0.05$) were considered statistically significant.

3. Results

There was no significant difference between the groups regarding the quantification of collagen in the tubulointerstitial compartment ($p = 0.716$), and the average percentage of collagen ranged from 0.11 to 0.76% between the groups. Moreover, the highest percentage of collagen in all the biopsies analyzed in this study was 3.92%. There was no significant difference between the groups concerning the evaluation of TGF-β1 in the tubulointerstitial and glomerular compartments. Moreover, there was only a mild immunoexpression in tubular cells and podocytes. On the other hand, there was a significant difference in the immunoexpression of TGF-β3 in the tubulointerstitial compartment between the groups; the Membranous Glomerulopathy Group and the control group had the lowest stainings, whereas the Berger's Disease Group had the highest staining (Table 1). Evaluation of this staining in the glomerular compartment showed a significant difference between the groups. The Lupus Nephritis and Acute Diffuse Glomerulonephritis (ADGN) Groups had a significantly higher immunoexpression of TGF-β3 than the control group (Fig. 1, Table 2).

There was a significant difference in fibronectin expression in the tubulointerstitial compartment between the groups; the Membranous Glomerulopathy Group and the control group had the lowest stainings, whereas the Lupus Nephritis and ADGN Groups had the highest stainings (Table 1). There was a significant difference between the groups regarding the expression of fibronectin in the glomerular compartment. The Podocytopathy (FSGS/MCD), Lupus Nephritis, ADGN and Membranous Glomerulopathy Groups had a significantly higher immunoexpression of fibronectin than the control group. Moreover, the Berger's Disease Group had a significantly lower immunoexpression of fibronectin