Expression of galectin-3 in nephrotic syndrome glomerulopathies in children

Danuta Ostalska-Nowicka¹, Michal Nowicki², Beata Kondraciuk², Malgorzata Partyka², Dariusz Samulak³, and Martin Witt⁴

Departments of ¹Paediatric Cardiology and Nephrology, ²Histology and Embryology, ³Surgical Gynaecology Poznan University of Medical Sciences, Poland, ⁴Anatomy Department, Technical University in Dresden, Germany

Abstract: Background: Galectins are a family of ancient animal carbohydrate binding proteins; the name is from their description as β-galactoside-specific lectins. They have been strongly implicated in inflammation and cancer. Studies of the association of galectins with various aspects of kidney disease in humans are still at an early stage. In line with the above, the aim of the present report was to analyse the immunohistochemical expression of galectin-3 (the only chimera galectin currently identified) in renal biopsy specimens of children with idiopathic nephrotic syndrome (INS). Patients and Methods: Eighteen children with minimal change disease (MCD), 30 with diffuse mesangial proliferation (DMP) and 11 with focal segmental glomerulosclerosis (FSGS) treated between 2003 and 2006 in the Department of Paediatric Cardiology and Nephrology, Poznan University of Medical Sciences. An indirect immunohistochemical protocol using a polyclonal rabbit antibody against human galectin-3 was employed. Results: In the control, MCD and DMP children who responded to steroid therapy anti-galectin-3 reactivity was present both in renal cortex and medulla. It was the strongest within cortical collecting ducts and subjectively less expressed in distal tubules. The total number of galectin-3 positive cortical and medullary segments of collecting ducts was significantly higher in the subjects who did not respond to steroid therapy These patients revealed also immunohistochemical reactivity of galectin-3 within nuclei of individual glomerular mesangial cells (p<0,001). Conclusions: A suggested galectin-3 authority in mature human glomeruli during proteinuric glomerulopathies may indicate, on the other hand, its anti-inflammatory effect, but on the other can prognosticate a further glomerular reconstruction leading to FSGS. Taken together, both glomerular and extraglomerular galectin-3 immunoreactivity in certain DMP individuals could be regarded as the factor of unfavourable prognosis.

Key words: galectin-3, glomerulopathy, tubulopathy, immunohistochemistry, children

Introduction

Galectins are a family of ancient animal carbohydrate binding proteins; the name is from their description as β-galactoside-specific lectins. They have been strongly implicated in inflammation and cancer and may be useful as targets for the development of new anti-inflammatory and anti-cancer therapies (reviewed in [1]).

Galectins are present at high concentration in a limited range of cell types, different for each galectin. They bind to sugar molecules on the surface of cells. All galectins bind lactose and other β-galactosides, but they differ in their affinity for more complex saccharides [2]. The galectins are defined by their structural similarities in their carbohydrate recognition domains (CRD) and by their affinity for β-galactosides. They have been classified into three classes, prototype, chimera, and tandem-repeat galectins (reviewed in [1]).

The only chimera galectin currently identified is galectin-3 which contains one CRD connected to a non-lectin domain; in addition to the CRD it contains a prolineand glycine-rich N-terminal domain (ND) through which is able to form oligomers [3-5]. Galectin-3 is also called Mac-2, L29, CBP35 or εBP, and is expressed in tumour cells (reviewed in [6]), macrophages [7], activated T cells [8], certain epithelial cells [9] and fibroblasts [10]. It binds a variety of matrix glycoproteins including laminin and
fibronectin [11]. Intracellularly, galectin-3 acts to prevent apoptosis [reviewed in [1]]. Depending on the cell type, galectin-3 can be localized in the extracellular matrix, the cell surface, in the cytoplasm, or in the nucleus [reviewed in [1]]. Galectin-3 has been shown to exhibit pro-inflammatory activities in vitro and in vivo; it induces pro-inflammatory and inhibits Th2 type cytokine production [reviewed in [1]]. High levels of circulating galectin-3 have been shown to correlate with the malignancy potential of several types of cancer. Galectin-3 is known to play a role in tumour growth, metastasis, and cell-to-cell adhesion [reviewed in [6]]. It also serves as a preferred substrate for matrix metalloproteinase-9 [12]. Finally, human and mouse galectin-3 share approximately 80% homology in their amino acid sequence [13].

The expression of galectin-3 is well known in animal urinary systems. In a normal rat kidney, it is expressed in epithelial cells of distal tubules [14,15]. Interestingly, in a rat model of acute mesangial proliferative glomerulonephritis [14], galectin-3 expression was found increased in distal tubules, as well as in glomerular macrophages, mesangial cells and proximal tubules. The similar pattern, except of mesangium and glomerular macrophages was observed in a ischaemia/reperfusion renal failure model in rats. Galectin-3 expression increased in proximal and distal tubuli at 2 – 48 h after reperfusion and was then followed by a normalization of the increased expression [15]. Therefore, those findings were considered to be acute reactions. On the other hand, in the study of streptozotocin-induced rat diabetes, galectin-3 expression in glomeruli was observed at 2 months after injury [16]. In the murine urinary system galectin-3 was expressed continuously from the kidney to the distal end of the urethra. The renal cortex expressed galectin-3 more intensely than the medulla. Renal galectin-3 immunoreactivity was strongest in the cortical collecting ducts, where principal cells were the sole cellular source.

Studies of the association of galectins with various aspects of kidney disease in humans are still at an early stage [17-20]. In children with primary glomerulonephritis, proteinuria is an important risk factor for the progression of glomerular disease and follows both qualitative and quantitative alterations in podocytes and the glomerular architecture [21]. The concept that podocytes, rather than mesangial cells, are the major culprit in the progression of glomerular diseases is gaining ground. In patients with nephrotic syndrome (NS), proteinuria (> 50 mg/kg per 24 h) and progressive loss of renal function are often mixed with tubular proteinuria superimposed on the underlying glomerular pattern [22]. This is most typical of focal segmental glomerulosclerosis (FSGS) with intensive proteinuria, progressive type [23]. The pathogenesis of the NS has an immunological basis and might be due to the presence of a circulating factor. This is entirely compatible with an immunological hypothesis, in that the factor(s) might be produced by cells of the immune system, either as the result of immunological reaction to antigen exposure or due to a primary abnormality of the immune system itself [24].

The aim of this study was to analyse the immunohistochemical expression of galectin-3 in renal biopsy specimens of children with idiopathic (primary) glomerulopathies.

Patients and Methods

Patients. The study group was composed of children with idiopathic nephrotic syndrome (INS), who were treated between 2003 and 2006 in the Department of Paediatric Cardiology and Nephrology, Poznan University of Medical Sciences. Two hundred and eighty-six children were referred to our clinic and evidence of proteinuria was found in 146 of them. Eighty-four patients (44 boys and 40 girls), following International Study for Kidney Diseases in Children recommendations, underwent a biopsy [25]. The indications included: primary idiopathic glomerulonephritis (n=36), steroid-resistant nephrotic syndrome (SRNS) (n=23) and secondary glomerulonephritis (n=25, who were excluded from further analysis). According to World Health Organization definitions [25,26], histological evaluation of the study group revealed: minimal change disease (MCD), n=18; diffuse mesangial proliferation (DMP), n=30; and FSGS, n=11. DMP diagnosis was based on the number of cells per mesangial area (four and more). INS was classically characterized by the absence of significant deposits by immunofluorescence microscopy (IF), except for FSGS and/or hyalinosis of FSGS, which bound IgM and C3 antiserum. There was no significant difference between the various categories defined by IF [26]. In 25 patients with DMP the presence of IgM and/or IgG within the mesangial area was observed without electron-dense deposits. No IgA nephropathy was diagnosed in the subjects. The relevant data are presented in Table 1.

The Ethics Committee of Poznan University of Medical Sciences approved the research protocol and the parents of all study participants gave informed consent for the investigation. Kidney biopsy specimens were fixed in Bouin’s solution for 24 h and subsequently embedded in paraffin.

Controls. Ten age- and sex-matched children in whom total nephrectomy due to the presence of Wilms tumour had been performed served as a control group. All of these subjects had normal renal function. Macroscopically normal-appearing kidney tissue was fixed in Bouin’s solution and saved for the subsequent procedure.

Antibodies and immunohistochemistry. For light microscopy, 6 μm thick sections were dewaxed and processed according to the indirect avidin-biotin-peroxidase technique [27]. A polyclonal rabbit antibody against human galectin-3 (diluted 1:100; Santa Cruz Biotechnology, sc-20157, Heidelberg, Germany) and monoclonal mouse anti-CD68 (diluted 1:100; Santa Cruz Biotechnology, sc-2072, Heidelberg, Germany) were employed. Endogenous peroxidase reaction was abolished by pre-treatment of the sections with 0.3% H2O2 in phosphate-buffered saline (PBS) prior to incubation with the antibodies. Following initial incubation with the primary antibody for 24 h at 4°C, sections were respectively incubated for 1 h at room temperature with goat anti-rabbit IgG (diluted 1:300; Sigma, Deisenhofen, Germany), goat anti-mouse IgG (diluted 1:300; Santa Cruz Biotechnology, sc-2072, Heidelberg, Germany)
and then incubated with a streptavidin-biotin-peroxidase reagent (Vectastain Elite; Vector Laboratories, Burlingame, CA, USA) for 45 min at room temperature. The activity of peroxidase was visualized using 0.5% 3,3’-diaminobenzidine (DAB) in Tris-HCl (pH 7.6) containing 0.3% H2O2. In all the control samples, the primary antibody was replaced by a non-immune serum or PBS. Photomicrographs were taken using a charge-coupled device (CCD) camera connected to a Nikon Digital Sight S-U1 unit, and the images were labelled and assembled as plates using Adobe Photoshop 7.0.

Double immunofluorescence. Some sections were double-labelled to confirm the identity of cells that were positive or negative for galectin-3 in the renal corpuscles. For this reason, a double immunofluorescence protocol was performed. Paraffin sections were dewaxed and incubated with mouse monoclonal anti-galectin-3 antibodies (diluted 1:10; Santa Cruz Biotechnology, sc-32790, Heidelberg, Germany) at 4°C overnight, followed by goat anti-mouse Alexa Flour 555 secondary antibody (1:200; Molecular Probes, MobiTec, Goettingen, Germany) at 37°C for 1 h. Subsequently, the sections were incubated with polyclonal rabbit anti-podocalyxin antibodies (1:600; a kind gift from Dr M. G. Farquhar, San Diego, CA, USA) at 37°C for 1 h, followed by incubation with Alexa Flour 488 goat anti-rabbit secondary antibody (1:200; Molecular Probes, MobiTec, Goettingen, Germany) for 30 min at 37°C. The sections were then mounted in buffered glyceringelatin. These were observed with an Olympus BX60 microscope. Pictures were taken using a CCD camera connected to Analysis Soft-Imaging System (Muenster, Germany).

Quantitative and statistical analysis. Due to the limited material provided by the clinical biopsies, we were not able to assess all serial sections of individual glomeruli. Instead, we measured the area of immunoreactive cells within a colour spectrum specific for the DAB staining and determined the value of at least five glomeruli in four subsequent sections as a percentage of the whole glomerular area. Based on the morphological heterogeneity of glomeruli, which displayed various stages of the respective pathological lesions, we analysed all available glomeruli which met either DMP or FSGS criteria (Table 1). Variations in antigen distribution within renal glomeruli were evaluated using Micro Image v.4.0 software (Olympus, MS Windows XP), measuring the area of marker expression on each colour image by using the ‘eye-dropper’ tool for each group of patients. Estimates of galectin-3 expression were subjected to mathematical analysis in order to examine changes of these markers in certain glomerulopathies. Differences between groups were then evaluated by one-way analysis of variance test (ANOVA). To determine the significance of between group means in the analysis of variance, the nearest significant difference test (Tukey test) was used as the multiple comparison analysis. Significance was set at p<0.05 [28]. Finally, the analysis was performed blind on coded samples.

Results

Clinical course

As regarded to the clinical course of nephrotic syndrome, there was no correlation between galectin-3 expression and age or sex. There were, however, significant differences in galectin-3 tissue localization between patients with proteinuria who did and did not respond to steroid therapy. In the former group, consisting of all MCD and DMP patients sensitive to steroids (DMP-s), galectin-3 was expressed within individual cortical collecting ducts and distal tubules as well as thin and thick limbs of loop of Henle and medullary collecting ducts. It was not present within renal corpuscles. The latter group of children, consisting of 12 DMP patients resistant to steroids (DMP-r) and all the FSGS children who developed an unfavourable clinical course of INS, revealed immunohistochemical expression of galectin-3 within all the mentioned above structures and, additionally, glomeruli (p<0.001). Detailed information is presented in Table 1.

Bright field microscopy

In the control and MCD children (Fig. 1a), anti-galectin-3 reactivity was present both in renal cortex and medulla. Within the cortical region it was the strongest within cortical collecting ducts and subjectively less expressed in distal tubules (Fig. 1b). Its expression was found within cell cytoplasm and nuclei of numerous, but not all, epithelial cells. The lining of proximal convoluted tubules softly expressed galectin-3. Glomerular area was free of galectin-3
reactivity (Fig. 1c). On the other hand, galectin-3 immunohistochemical presence was also found in both thin and thick limbs of loop of Henle and collecting ducts within renal medulla (Figs. 2a-2c). The same galectin-3 pattern was observed in all the DMP-s children. (Figs. 1d-1f). However, in subjects who did not respond to steroid therapy (both DMP-r and all the FSGS), the total number of galectin-3 positive cortical and medullar segments of collecting ducts (counted as a number of cross-sected structures per square area)
was significantly higher (p=0.0045; Figs. 1g, 1h, 1k and 1l). Interestingly, these patients revealed also immunohistochemical reactivity of galectin-3 within nuclei of individual mesangial cells (p<0.001, Figs. 1i and 1m). CD68 was primarily found in areas of the interstitial inflammatory infiltration (Fig. 3a) suggesting the presence of macrophages. Its localisation, however, was independent from age, sex, or type of glomerulopathy and was never followed by co-existence with galectin-3 (Figs 3a-3d).

**Double immunofluorescence microscopy**

In double incubations with anti-galectin-3 and anti-podocalyxin the analysed markers did not reveal co-localization in renal glomeruli (Fig. 4). The expression of podocalyxin was present in podocytes of all the children. Galectin-3 was not expressed in intraglomerular structures of controls, MCD and DMP-s (data not shown). Its expression was exclusively present in the nuclei of individual cells (mesangial cells) situated in the closest proximity of glomerular capillaries of DMP-r and FSGS subjects who did not respond to steroid therapy.

**Discussion**

Functional properties of galectin-3 strongly suggest its importance in the regulation of the immune response and inflammation (review in [1]). In general, galectin-3 is a powerful pro-inflammatory signal protein. Certain cells produce and secrete a large amount of galectin-3 as a response to various inflammatory stimuli. When secreted or externalized, galectin-3 may affect inflammatory cells by an autocrine or paracrine mechanism [review in [6]]; it promotes adhesion of human neutrophils to laminin [29] as well as endothelial cells [30] and acts as a chemoattractant for monocytes and macrophages [31]. Galectins, in general, are not expressed or expressed at lower levels in resting lymphoid cells [8,32,33]. Infection and transformation of T cells with human T lymphotropic virus-1 or human immunodeficiency virus type 1 result in increased levels of galectin-3 [34]. Interestingly, galectin-3, which was previously known as Mac-2, could be identified on the cell surface of mouse peritoneal macrophages, and is considered as a marker for certain mature macrophages [7]. In humans, the galectin-3 level increases dramatically when monocytes differentiate into macrophages [35]. It is also expressed in mast cells [36], neutrophils [37] and eosinophils [38]. In line with this, galectin-3 is suggested to be a positive regulator of T-cell growth [39]. What is more, galectin-3 was also documented as an anti-apoptotic factor [40,41]. It has been found to interact with a number of proteins implicated in apoptosis regulation (Bcl-2, Fas receptor) [41,42]. It appears that galectin-3 localized in the cytosol protects the cell from apoptosis, but localized in nucleus has an opposite effect (reviewed in [1]).

Results of the present report have to be discussed in two different aspects. First, as intraglomerular galectin-3 expression; secondly as its presence in extraglomerular area (both cortical and medullar).

Glomerular expression of galectin-3 was found exclusively in a few FSGS and DMP proteinuric subjects who did not respond to steroid therapy. According to the double immunofluorescence assay and the subsequent incubations with CD68, the cells reactive for galectin-3 were recognized as mesangial cells. They were not co-localised with podocalyxin – a
marker of podocyte foot processes surface surrounding capillaries [20] and were situated outside the blood vessels. That localisation refers to another remarks of galectin-3 distribution observed in a rat model of acute mesangial proliferative glomerulonephritis [14]. Interestingly, galectin-3 positive mesangial cells in FSGS children were present exclusively within approx. 15% of glomeruli which did not reveal sclerotic features. The rest of glomeruli (85%) was galectin-3 negative. It is in line with our another observation where four of DMP-r patients have underwent the kidney re-biopsy within 3 years of observation; all of these were diagnosed at that moment with FSGS and, curiously, had galectin-3 immuno-negative glomerular pattern.

In the study demonstrated by Kikuchi Y et al. the number of galectin-3-positive cells in human diabetic glomeruli significantly increased compared with other glomerular diseases [18]. In that report, most galectin-3-positive cells in all glomerular disorders were also stained with CD68 and HLA-DR, suggesting that these cells are activated macrophages [18]. It was also insinuated that infiltrated macrophages in kidneys might play an important role in the progression of IgA nephropathy, crescent glomerulonephritis and other renal diseases [43,44]. Moreover, the glomerular infiltration of macrophages in diabetic kidneys was thought also to play a crucial role in the progression of nephropathy. Our results indicate that both glomerular or extraglomerular galectin-3 positive cells were never co-expressed with CD68. This may suggest that, contrary to the general point of view, activated macrophages do not always need express galectin-3. Interestingly, glomerular galectin-3 was expressed exclusively in the nuclei of mesangial cells suggesting its pro-apoptotic role. In our previous study involving galectin-1 expression in childhood glomerulopathies, it was detected in renal podocytes in DMP and FSGS cases, while control glomeruli and MCD were negative [20]. Galectin-1 immunoreactivity was found also within parietal epithelial cells in patients with FSGS. These results suggested a possible role for galectin-1 in the pathogenesis of primary glomerulopathies in children as a kind of podocyte-related self-protective activity and probably involvement of epithelial cells of Bowman's capsule in inflammatory processes [20]. It seems to be possible that the similar action implied in our present report might insinuate a similar self-protective activity according to mesangial cells. On the other hand, it should result in a more favourable clinical course. Why then these patients developed resistant to steroids proteinuria?

This apparent discrepancy might not be logically explained unless we comment a second aspect of galectin-3 expression present in collecting ducts, loop of Henle and distal tubules. That localisation of galectin-3 and its possible significance in the urinary system was primarily described on animal model. It is generally believed that the expression of galectin-3 in the kidney occurs predominantly during early developmental stages [45,46]. The expression level in the developing mouse kidney is reduced sharply after birth, suggesting that the main role of galectin-3 is related to nephrogenesis; for example, galectin-3 functions as a negative regulator of growth and maturation.
Expression of galectin-3

Fig. 4. Double incubation of galectin-3 (red) and podocalyxin (green) in glomerulus of a 7-year-old child with resistant to steroids diffuse mesangial proliferation (scale bar 25 μm). The expression of podocalyxin is present in podocytes (marker of podocyte foot processes surface surrounding capillaries). Galectin-3 is expressed in the nuclei of individual mesangial cells situated in the closest proximity of glomerular capillaries as well as extra-glomerular tubular cells.

of the uretic tree [45,47] or, as described above, pro-apoptotic factor. Single remarks indicate also a considerably high expression level of galectin-3 in the mouse adult kidney at the mRNA and protein levels [48]. Immunohistochemically, it was predominantly expressed in the cortical collecting ducts and the connecting segments. The galectin-3 expression in the renal medulla of adult mice was less intense and was mainly localized in the medullary and papillary collecting ducts. This was in contrast to the finding in the developing human kidney, where the most intense immunoreactivity for galectin-3 was found in medullary and papillary collecting ducts rather than cortical collecting ducts [49].

In our study, a significantly higher number (density) of immunopositive tubules (loop of Henle, distal tubules and collecting ducts) was found in steroid-resistant individuals (Fig. 1g and 1k). A 'tiger-like' pattern of galectin-3 immunoreactivity in cortical collecting ducts and distal convoluted tubules with its subsequent expression in cytoplasm or nuclei (Fig. 1i and 1m) we would brave to interpret as a new DMP marker. Why these structures are galectin-3 negative. Taken together, both glomerular and extraglomerular galectin-3 immunoreactivity in certain DMP individuals could be regarded as the factor of unfavourable prognosis.

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