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IMMUNOHISTOLOGIC KIDNEY BIOPSY STUDY OF BALKAN ENDEMIC NEPHROPATHY

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Summary. *The pathogenesis of renal histologic changes in Balkan endemic nephropathy (BEN) is still insufficiently understood. We studied the deposition of immune reactants in correlation with tubulo-interstitial histologic changes and the degree of proteinuria in 50 kidney biopsies of BEN patients and expression of collagen IV, laminin, vimentin and smooth muscle actin in 5 biopsies. The immunofluorescence technique, using antibodies for IgA, IgG, IgM, kappa and lambda light chains, complement components C3, C1q and C4, fibrin/fibrinogen, and albumin, as well as the streptavidin-biotin peroxidase technique, using antibodies for collagen IV, laminin, vimentin and smooth muscle actin were applied. Glomerular and vascular staining for immune reactants was mainly negative or insignificant. Deposits of C3 along the tubular epithelium correlated with the intensity of tubulo-interstitial changes and the degree of proteinuria. Resorptive droplets of mainly albumin and immunoglobulin light chains in proximal tubules were less frequent in cases with more severe tubular changes and higher proteinuria. Collagen IV and laminin were overexpressed in interstitial capillaries and the surrounding interstitium, occasionally associated with smooth muscle actin positive interstitial cells. Vimentin was coexpressed in the epithelium of injured tubuli. In conclusion, humoral immune mechanisms do not appear to play a pathogenetic role in BEN. Interstitial sclerosis seems to result from the overproduction of the basement membrane-related extracellular matrix by injured and activated tubular epithelial cells and interstitial capillary endothelial cells, as well as production of connective tissue fibers by proliferating myofibroblasts.*

Key words: *Balkan endemic nephropathy, kidney biopsy, immunohistology, pathogenesis*

Introduction

The etiology and pathogenesis of Balkan endemic nephropathy (BEN) are still unknown. A limited number of early immunohistologic and histologic studies of BEN suggested immune and autoimmune reactions as a possible pathogenetic mechanism (1-5). In contrast, other studies (6-9), including our previous study (10), did not confirm this due to negative or presumably non-specific immunohistologic findings presented mainly as positive staining for IgM and complement C3 in sclerosed glomeruli and blood vessels.

Evidence has accumulated that tubular epithelial cells probably play a key role in progressive loss of kidney function in various pathologic conditions. Initial injury or activation of tubular epithelial cell by different mechanisms can consequently induce enhanced local production of complement, chemokines, cytokines, as well as extracellular matrix components, leading to irreversible interstitial injury and loss of renal function (11). Tubular deposits of complement C3 were actually found in 16% of kidney biopsies of BEN patients in our previous study (10), and interstitial sclerosis, suggesting the enlargement of the extracellular matrix rather than fibrosis, was described in early studies as a characteristic feature of BEN (12-13).

In this study, deposition of immune reactants in the tubulo-interstitial compartment was compared with tubulo-interstitial histologic changes and the degree of proteinuria. Furthermore, a preliminary immunohistochemical localization and evaluation of selected extracellular matrix components and cytoskeletal proteins was introduced.

Material and Methods

Renal tissue samples from 52 patients with BEN in early stages from the endemic area northeast of Bosnia were available for immunofluorescence microscopy study. The group consisted of 13 males and 39 females, with an age range of 27 to 64 years and a mean age of 46.9 ± 2.5 years. Clinical data and detailed evaluation of histologic renal changes in this group of BEN patients have been reported elsewhere (14-15).

For immunofluorescence microscopy examination, renal tissue samples were transported in Histocon and within three days quick-frozen in liquid nitrogen. Five μm cryostat sections were incubated with fluorescein isothiocyanate (FITC) labelled antisera to human IgA, IgG and IgM, kappa and lambda light chains, comple-

ment components C3, C1q and C4, fibrin/fibrinogen and albumin (Dako, Denmark), according to previously described standard procedures of our laboratory (10). Glomerular, extraglomerular vascular and tubulo-interstitial deposits of immune reactants were assessed and semiquantitatively scaled from 0 to 4+ in the previously reported study (10). In the present study, including 50 patients, the emphasis was given to evaluation of immune reactant deposits in the tubulo-interstitial compartment and their comparison with tubulo-interstitial histologic changes and the degree of proteinuria.

Furthermore, a preliminary immunohistochemical study was performed which applied the streptavidin-biotin peroxidase technique on formalin-fixed Paraplast embedded tissue samples of 5 biopsies using the Dako protocol for antigen retrieval and the Dako protocol for an optimized staining system on automatic immunostainer Dako TechMate TM 500 (Dako, Denmark). The following primary antibodies were used: mouse monoclonal antibodies against vimentin, smooth muscle actin (Dako, Denmark) and laminin (Sigma, U.S.A.) and rabbit polyclonal antibody against collagen IV (Dako, Denmark).

Statistical analysis included the chi-square test for comparison of the proportions. $P<0.05$ was taken to be significant.

Results

Detailed immunofluorescence findings are shown in Table 1. Nonsclerosed glomeruli were mostly negative or revealed scanty focal segmental granular deposits of immune reactants. The exception were 5 cases with diffuse moderate, predominantly mesangial IgA deposits associated with mild mesangial proliferation, and 5 cases with predominant IgM, present globally or segmentally in the glomerular capillary walls, which were by light microscopy thickened and showed a double-outline of the glomerular basement membrane. A diagnosis of co-occurring IgA nephropathy was established in patients with mesangial IgA deposits and thrombotic microangiopathy-like histologic changes were described in cases with capillary wall IgM deposits. Lumpy deposits of IgM and C3 were found in sclerosed glomeruli as well as in small extraglomerular vessels expressing sclerosis and hyalinosis. Granular deposits of complement C3 on the outer aspect of tubular epithelial cells, most probably of proximal tubules, were observed in 8 (16.0%), and resorptive droplets, containing predominantly albumin and immunoglobulin light chains were present in proximal tubules of 20 (40.0%) cases.

We compared tubular C3 deposits and tubular resorptive droplets with semiquantitatively assessed tubulo-interstitial changes (Table 2). Tubular deposits of complement tended to be associated with more intensive tubulo-interstitial changes, while resorptive droplets in epithelial cells of proximal tubules were more frequently present in cases with milder histologic changes. A similar relationship was established between tubular deposits of C3 and resorptive droplets on the one hand and the degree of proteinuria on the other (Table 3). Differences were not statistically significant, except for the correlation between tubular resorptive droplets and proteinuria ($P = 0.001$). If we joined the two groups of patients with proteinuria <300 mg/l, then the correlation between tubular C3 deposits and proteinuria approached statistical significance ($P = 0.088$).

Table 1. Immunohistologic findings in kidney biopsies of 52 BEN patients

Location of immune deposits	Composition of immune deposits								
	IgA	IgG	IgM	κ	λ	C3	Clq	C4	Alb
Glomeruli	11*	3	16	11	11	15	2	1	9
Vessels	0	0	9	0	0	45	3	1	0
Tubuli	0	0	0	0	0	8	0	0	8
Resorptive droplets	2	2	0	11	11	0	0	0	17

* Moderate granular mesangial IgA deposits in 5 patients fulfilled the criteria for the diagnosis of IgA nephropathy

Table 2. Comparison of tubular C3 deposits and resorptive droplets with semiquantitatively assessed tubulo-interstitial changes in 50 BEN patients

Tubulo-interstitial changes	No. of cases	Tubular C3 deposits	Tubular resorptive droplets
Null, mild	36	4 (11.1%)	17 (47.2%)
Moderate, severe	14	4 (28.6%)	3 (21.4%)
P value		0.131	0.095

Table 3. Comparison of tubular C3 deposits and resorptive droplets with proteinuria in 50 BEN patients

Proteinuria (SDE)	No. of cases	Tubular C3 deposits	Tubular resorptive droplets
< 150 mg/l	22	2 (9.1%)	15 (68.2%)
150-300 mg/l	10	1 (10.0%)	2 (20.0%)
>300 mg/l	18	5 (27.7%)	3 (16.7%)
P value		0.234	0.001

Our preliminary immunohistochemical study of selected extracellular matrix components and cytoskeletal proteins localization included 5 biopsies of BEN patients. Overexpression of collagen IV and laminin within the thickened tubular basement membrane of atrophic tubuli and particularly in renal interstitial capillaries and the surrounding widened interstitium was established. Increased collagen IV and laminin expression was noticed also in the sclerosed glomeruli and sclerosed segments of glomeruli. Vimentin was expressed in non-sclerosed glomeruli, particularly podocytes, the interstitium and blood vessels and in addition, focally, more or less extensively coexpressed with cytokeratins in the epithelial cells of injured tubuli, while unaffected tubuli appeared negative. Smooth muscle actin expression was noticed in the glomerular mesangium and blood vessel walls and, occasionally, there was also focal marked expression in the widened interstitium.

Discussion

In our previous detailed immunofluorescence microscopy study of kidney biopsies of 52 BEN patients, we observed as the most frequent finding nonspecific scanty, often segmental glomerular deposits of IgM and/or C3 or negative immunofluorescence (10). More intensive lumpy immunostaining for IgM and complement components in sclerosed glomeruli and blood vessels were also assumed to result from nonspecific protein entrapment at sites of sclerosis. Furthermore, segmental or global capillary wall deposits of IgM and/or C3 found associated with chronic thrombotic microangiopathy-like glomerular lesions suggested endothelial cell injury and secondary plasma protein insudation. We concluded that patterns of immune reactant deposition in BEN are mostly nonspecific and do not reveal humoral immunity as a pathogenetic mechanism of the disease. However, 5 out of 52 BEN patients had diffuse predominant granular mesangial IgA deposits accompanied by complement C3. It is well known that this pattern is characteristic of IgA nephropathy. We assumed that these patients presented the coincidence of BEN and clinically mild IgA nephropathy, particularly since IgA nephropathy is widely recognized to be by far the most frequent form of immune complex mediated glomerulonephritis.

In this study, special emphasis was given to comparison of tubular epithelial cell resorptive droplets and tubular deposits of C3 with tubulo-interstitial histologic changes and proteinuria. The filtration of low-molecular-weight proteins through the glomerulus, and consequent resorption and degradation by tubular epithelial cells, is a normal process. We found resorptive droplets, containing particularly low-molecular-weight proteins such as albumin and immunoglobulin light chains, in the epithelial cells of proximal tubules less frequently in BEN patients with more severe tubulo-interstitial histologic changes and a higher degree of proteinuria. It can be assumed that proximal tubules are impaired in BEN and as a consequence, their capacity for protein resorption is reduced.

Furthermore, evidence has emerged, mostly from experimental studies, that the proximal tubular cells have an important role in the progression of renal disease, irrespective of the causal factor (11,16). Following initial injury, tubular epithelial cells become activated and produce inflammatory mediators like cytokines, chemokines, complement as well as growth factors and matrix components. In our study, tubular deposits of complement C3 were more frequently observed in association with more intense tubulo-interstitial histologic changes and a higher degree of proteinuria. This finding could indicate enchanced local production of complement by injured and activated tubular epithelial cells in BEN.

A significant role of tubular injury in the pathogenesis of BEN was indicated not only histomorphologically but in this study also by the finding of vimentin and cytokeratins coexpression in the tubular epithelium. According to the study of Gröne et al (17), coexpression of keratin and vimentin is characteristically found in damaged and regenerating tubular epithelia of the kidney, irrespective of the cause of injury. A similar observation in BEN has already been published by Stefanović et al (18).

We found in our BEN patients overexpression of collagen IV and laminin in thickened tubuli and, particularly, interstitial capillaries. This is complementary to the results of immunohistochemical localization of laminin in kidney of BEN patients studied by Čukuranović et al (19). Basement membrane overproduction and its participation in the development of sclerosis characteristic of BEN may result from injury by an unknown agent and secondary overactivity of both tubular epithelial and capillary endothelial cells. Interstitial cells coexpressing vimentin and smooth muscle actin demonstrated in our study may suggest, in addition, myofibroblast proliferation and their participation in the increased production of interstitial extracellular matrix collagens in BEN. A similar suggestion was already provided by our previous electron microscopy study, which revealed an increased deposition of basement membrane-like material closely associated with thickened and split basement membranes of the atrophic tubuli and sclerosed interstitial capillaries (20,21).

In summary, humoral immune mechanisms do not appear to be involved in the pathogenesis of BEN. However, immunofluorescence examination of kidney biopsy is mandatory to exclude immune mediated diseases in endemic areas and recognize possible concomitant immune mediated forms of glomerulonephritis. Our preliminary immunohistochemical study confirmed that interstitial sclerosis in BEN is not only the result of an increase of the connective tissue fibres but particularly the result of an increased deposition of extracellular basement membrane-related matrix. Furthermore, evidence exists that an unknown agent may cause simultaneous injury of tubuli and small intrarenal vessels, including interstitial capillaries, in the pathogenesis of BEN.

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