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Original contribution

Interstitial inflammation in Alport syndrome☆

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Summary The Alport syndrome is a hereditary glomerular disease linked to structural abnormalities of collagen IV. In a mouse model of Alport syndrome, the interstitial lymphocyte influx was important for disease progression. CXCR3 is a chemokine receptor involved in lymphocyte recruitment to the kidney. We hypothesized that CXCR3-positive T cells might be involved in human Alport syndrome. Immunohistochemistry was performed on formalin-fixed, paraffin-embedded biopsies from 17 patients with Alport syndrome, 10 with immunoglobulin A (IgA) nephropathy, and 11 healthy donor kidneys. We investigated the expression of the α5 chain of collagen IV to confirm the morphologic diagnosis, the chemokine receptor CXCR3 and CD3-positive T cells. Alport syndrome biopsies demonstrated a complete loss of the α5 chain of collagen IV from the glomerular basement membrane and the morphologic features consistent with Alport syndrome on electron microscopy. A prominent number of CXCR3-positive cells were found in the tubulointerstitium. Most of the CXCR3-positive cells were CD3-positive T cells, demonstrated by double-labeling in selected biopsies. The number of CXCR3-positive cells in kidneys with Alport syndrome correlated with serum creatinine ($P < .05$) and with morphologic features of a progressive disease (eg, interstitial fibrosis, glomerulosclerosis, and tubular atrophy). The severity of interstitial CXCR3-positive cell influx was similar in Alport syndrome as compared to immunoglobulin A nephropathy. The noninflammatory glomerular lesion of Alport syndrome is associated with prominent interstitial accumulation of CD3- and CXCR3-positive lymphocytes. The degree of infiltration correlated with renal function. We speculate that targeting T lymphocytes, for example, by CXCR3 blocking agents, might be a novel approach to inhibit disease progression in patients with Alport syndrome.

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1. Introduction

Alport syndrome (AS) is a rare hereditary kidney disease, representing 0.3% to 2.3% of the patients on renal replacement therapy [1-3]. It is caused by mutations of collagen type IV, which is an essential component of glomerular basement membranes [4]. Collagen IV consists of triple helices of different α chains [5]. The α3α4α5 variant is expressed in the basement membranes of glomeruli, Bowman capsule, the cochlea, and the lens [6]. A defect in one of the genes of the α3α4α5 trimer is sufficient to cause AS and the most common form results from mutations of the α5 gene localized on the X chromosome [7].

During the last decades, the genetic causes and the glomerular alterations in AS have been extensively studied [2,4]. In contrast, tubulointerstitial infiltrates have not been well characterized. This is unexpected as the early literature mentioned not only a marked accumulation of fat-laden macrophages ("foam cells") but also prominent accumulation of infiltrating lymphocytes [8]. In post-mortem specimens, "numerous large foci of lymphocytes" were described [8]. Terms like *chronic pyelonephritis*, *mixed nephritis*, *inherited pyelonephritis*, and *inherited interstitial nephritis* were a reflection of significant inflammatory lesions in the tubulointerstitial space [9,10]. It even resulted in a discussion whether the primary lesion was glomerular or interstitial.

The collagen α3–deficient mice have been extensively used as a model of human AS [11-14]. Lymphocyte deficiency resulted in a significant reduction of interstitial injury and improvement in disease progression in the α3-deficient mice [15]. The recruitment of inflammatory cells into the kidney is orchestrated in part by a large family of small chemotactic cytokines, called the chemokines [16,17]. CXCR3 is a chemokine receptor that is primarily expressed on activated T lymphocytes and is important in lymphocyte recruitment to the kidney [18,19]. We have previously shown that CXCR3-positive T cells form a significant part of the interstitial infiltrate in the most common forms of glomerulonephritis [20]. The hypothesis of the present study was that a noninflammatory glomerular damage, which is caused by mutations of type IV collagen in AS, triggers the recruitment of inflammatory cells to the tubulointerstitium. This might then promote progressive remodeling of the interstitial compartment, that is, tubular atrophy and interstitial fibrosis. As a first step, we localized CXCR3-positive cells and T cells in a cohort of patients with AS. We found a high number of CXCR3-positive cells in the tubulointerstitium, which at times demonstrated tubular aggression ("tubulitis"). Of importance, the degree of CXCR3-positive T-cell infiltration was inversely correlated with renal function at the time of biopsy.

2. Materials and methods

A total of 38 archival formalin-fixed, paraffin-embedded renal specimens were included in the quantitative study. Biopsies were from patients with AS (n = 17), with immunoglobulin A (IgA) nephropathy (n = 10), and pretransplant biopsies from donor kidneys as controls (n = 11). The morphologic diagnosis of AS was based on routine light microscopy, immunohistochemistry, and electron microscopy. All patients with AS demonstrated glomerular basement membrane changes on electron microscopy. Severe thinning of the basement membrane was present in 8 specimens. In addition, all specimens demonstrated areas of basement membrane splitting. In 2 biopsies, splitting of the basement membrane was scored as mild; in the other cases, it was found to be prominent. The diagnosis was confirmed by a complete loss of the glomerular collagen α5 chain in these biopsies. One additional biopsy diagnosed as AS in the same way was only used for double-labeling but was not included in the quantitative analysis (because of scarcity of the biopsy material). A group of patients with IgA nephropathy (n = 10) was included for the comparison of the severity of the tubulointerstitial lesions. The light microscopic features (eg, interstitial fibrosis, mesangial sclerosis, mesangial cellularity) were scored semiquantitatively from 0 to 4 by an observer blinded to the results of the CXCR3 cell number quantification. This retrospective study was performed on archival material after the diagnostic studies were finalized for which the Austrian legislation does not require patient consent.

2.1. Immunohistochemistry

Immunohistochemistry was performed as previously described [20,21]. In brief, slides were dewaxed in xylene and rehydrated in a graded series of ethanol. Endogenous peroxidases were blocked in 0.3% H_2O_2 and endogenous biotin by the Avidin/Biotin blocking kit (Vector Laboratories, Burlingame, CA). The antigen retrieval was performed in an autoclave oven. For CD3 or CXCR3, the antigen unmasking solution (Vector Laboratories) was used. For the α2, α3, or α5 chain of the collagen IV, the slides were heated in HCl (pH 0.9). Antibodies were diluted in 10% nonfat dry milk (Sigma-Aldrich, Taufkirchen, Germany). Slides were incubated with a superblock liquid (IDLabs Inc., London, ON, Canada) for 10 minutes. Primary antibodies were used for 60 minutes. Secondary and tertiary biotinylated antibodies were incubated for 30 minutes each, as well as the final incubation with the Vectastain Elite ABC Kit (Vector Laboratories). As the detection system, we used 3,3′-diaminobenzidine (Sigma-Aldrich) combined with $NiCl_3$ enhancement (which causes the precipitate to turn black). Slides were dehydrated, counterstained with methyl-green, and mounted with Vectamount (Vector Laboratories).

As primary antibodies, we used a rat antihuman CD3 monoclonal antibody (IgG1, Clone CD3-12, AbD Serotec, Planegg/Martinsried, Germany), mouse antihuman CXCR3 monoclonal antibody (IgG1, κ, Clone 1C6, BD Biosciences Pharmingen, Heidelberg, Germany), monoclonal mouse anti-CD68 (Clone PG-M1, DAKO Germany, Hamburg, Germany), and noncommercial rat antihuman α2 (H22), α3