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Upregulation of c-mip is closely related to podocyte dysfunction in membranous nephropathy

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Membranous nephropathy is a glomerular disease typified by a nephrotic syndrome without infiltration of inflammatory cells or proliferation of resident cells. Although the cause of the disease is unknown, the primary pathology involves the generation of autoantibodies against antigen targets on the surface of podocytes. The mechanisms of nephrotic proteinuria, which reflect a profound podocyte dysfunction, remain unclear. We previously found a new gene, *c-mip* (c-maf-inducing protein), that was associated with the pathophysiology of idiopathic nephrotic syndrome. Here we found that c-mip was not detected in the glomeruli of rats with passive-type Heymann nephritis given a single dose of anti-megalin polyclonal antibody, yet immune complexes were readily present, but without triggering of proteinuria. Rats reinjected with anti-megalin develop heavy proteinuria a few days later, concomitant with c-mip overproduction in podocytes. This overexpression was associated with the downregulation of synaptopodin in patients with membranous nephropathy, rats with passive Heymann nephritis, and c-mip transgenic mice, while the abundance of death-associated protein kinase and integrin-linked kinase was increased. Cyclosporine treatment significantly reduced proteinuria in rats with passive Heymann nephritis, concomitant with downregulation of c-mip in podocytes. Thus, c-mip has an active role in the podocyte disorders of membranous nephropathy.

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Idiopathic membranous nephropathy (MN) is a glomerular disease of unknown etiology, commonly associated with nephrotic syndrome. It is histologically characterized by diffuse thickening of the capillary loop, which results from the formation of subepithelial immune deposits consisting of immunoglobulin G (predominantly IgG4), complement components, and recently identified antigens.[1–4] MN remains to be a major cause of nephrotic syndrome in adults, with up to 40% of patients progressing toward end-stage renal failure after 10 years.[5] Researches into the molecular mechanisms underlying the pathogenesis of MN were focused many years ago on the identification of target antigens, which initiate the formation of the immune complex. The generation of an experimental model of MN established in rats by Heymann *et al.*[6] 50 years ago represents the start point of pathophysiological researches on MN. Active- (AHN) and passive-type Heymann nephritis (PHN) were induced by direct immunization of Lewis rats with a crude preparation of brush border proteins or by injection of rabbit anti-rat brush border antibodies, respectively. Both AHN and PHN closely mimic the human glomerular disease.[7] The identification in this model of the target antigen, megalin, a common component of the tubular and glomerular epithelial cell, provided the molecular basis of podocyte disease in MN.[8,9] Nevertheless, as human podocytes do not express megalin, it cannot be considered as the target antigen in human MN.[1] In the past decades, many studies have focused on the identification of the antigen involved in human MN. Several antigens involved in secondary forms of MN caused by infectious disease and cancer have been found in subepithelial immune deposits, without clear evidence for a direct pathogenic role. The first identification of a pathogenic antigen in human MN came from Ronco *et al.*,[10,11] who identified neutral endopeptidase (NEP) as the podocyte target of nephritogenic antibodies in patients with neonatal MN. The anti-NEP antibodies resulted from immunization

against the placental NEP of an NEP-deficient mother. In this form of MN, the membrane attack of complement (C5b–9) was also detected within the immune deposits, which indicated that, similar to anti-megalin antibodies, anti-NEP could induce complement activation, potentially leading to podocyte injury.[11,12] Subsequently, Beck *et al.*[3] reported that about 70% of patients with idiopathic MN have autoantibodies that react with the M-type phospholipase A2 receptor (PLA2-R), a glycoprotein constitutively expressed in podocytes. Prunotto *et al.*[4] identified two additional targets of autoimmunity, aldoreductase and SOD2, both induced in glomeruli of patients with MN.

The onset of proteinuria during the course of MN is associated with phenotypic alteration of podocyte and slit diaphragm integrity, as demonstrated in a Heymann nephritis model.[13–15] The abundance of nephrin, a major protein of slit diaphragm, is reduced in PHN and in biopsies from patients with MN, whereas the mRNA and protein expression of cation-permeable ion channel TRPC6, which is mutated in the familial form of focal segmental glomerulosclerosis,[16] is increased.[13–15,17,18] However, the mechanisms that lead to these alterations remain partly obscure.

The membrane attack complex of complement C5b–9/Mac is thought to have a crucial role in the induction of podocyte injury leading to proteinuria in this model.[12,19] In PHN, proteinuria has been suggested to be entirely dependent on C5b–9/Mac.[20] These data have recently been challenged by the finding that AHN and PHN can be induced in a rat deficient in complement component C6, which is unable to form Mac.[21,22] Indeed, in PVG/C6$^{-/-}$ rats, the degree of proteinuria, the extent of cellular infiltrates, and the abundance of Ig, C3, and electron-dense deposits were similar to those observed in the PVG/c rats.[22] Anti-podocyte antibodies including anti-megalin, anti-NEP, and anti-PLA2-R are directed against functional receptor or enzymatic proteins, so that their binding may induce a cascade of events that may directly alter podocyte biology.[23]

In the wake of studies aimed to understand the molecular pathophysiology of idiopathic nephrotic syndrome, we identified a new gene, *c-mip* (for c-maf-inducing protein), which encodes an 86-kDa protein.[24] The predicted structure of c-mip includes an N-terminal region containing a pleckstrin homology domain, a middle region characterized by the presence of several interacting docking sites, including a 14-3-3 module, a PKC domain, an Erk domain, a SH3 domain similar to the p85 regulatory subunit of phosphatidylinositol 3-kinase, and a C-terminal region containing a leucin-rich repeat domain.

We have recently shown that c-mip abundance is increased in MN during relapse,[25] which led us to study its potential implication in Heymann nephritis. We report here that c-mip protein is not induced at the early stage of PHN, when the immune complex deposits are formed without inducing proteinuria, but increases very quickly after a second injection of anti-megalin polyclonal antibodies, while proteinuria concomitantly rises to reach nephrotic range. We provide evidence that c-mip induces *in vivo* and *in vitro* podocyte dysfunctions that are common to MN and PHN.

RESULTS

Renal expression of c-mip in MN and PHN

Northern blot analysis showed that basal expression of *c-mip* in podocyte was scarcely or below the detection limits in control human kidneys (Figure 1a), which suggests that *c-mip* is transcriptionally repressed in physiological situations. However, quantitative PCR (qPCR) from laser-microdissected glomeruli from 5 control samples and 11 MN biopsy specimens showed that *c-mip* abundance was significantly increased in MN (Figure 1b). In addition, we confirmed by *in situ* hybridization (Figure 1c), confocal immunofluorescence (Figure 1d), and immunohistochemistry analysis (Supplementary Figure S1 online) that c-mip was overproduced at the mRNA and protein levels in patients with MN.

The finding that c-mip was highly induced in podocytes of patients with MN led us to study its expression in Heymann nephritis, the experimental model of human MN. We induced PHN by injection of anti-megalin polyclonal antibody. Proteinuria, as tested at day 13 post injection, was very slightly increased (urine protein to creatinine ratio, UPr/UCr, mg/mg ± s.d.: 1.53 ± 0.20) relatively to controls (0.63 ± 0.057) (Figure 2a). At day 12, immunofluorescence analysis of kidney sections showed granular deposits of IgG along the glomerular capillary loops in rats with PHN, whereas no staining was visualized in control rats (Figure 2b). Following a second injection 2 weeks after the first one (day 14), rats developed heavy proteinuria that reached a peak at day 19 (UPr/UCr: 9.48 ± 7.64) (Figure 2a). Quantitative reverse transcription (RT)-PCR from laser-microdissected glomeruli ($n = 3$ rats at each time point) showed that c-mip abundance was markedly increased 24 h after the second immunization (Figure 2c). c-mip was visualized by immunohistochemistry by day 13 post injection and increased much at day 15, whereas no signal was detected before (Figure 2d). Overproduction of c-mip persisted until day 42, along the experimental procedure. These results suggest that c-mip induction is paralleled to the development of proteinuria.

Overexpression of c-mip induces phenotypical and biochemical alterations

To understand the effects of c-mip on podocyte function in PHN, we established stably transfected podocyte cell lines using the inducible T Rex system. Stable c-mip-overexpressing cells exhibited abnormal morphology with retraction of cell body, loss of stress fibers, and were more susceptible to cell detachment from collagen matrix, whereas podocytes cultured in the absence of tetracycline proliferate normally and exhibited long intracellular bundles of actin filaments (Figure 3a and Supplementary Figure S2 online). The abundance of synaptopodin, a regulator of podocyte actin cytoskeleton reorganization, was decreased (Figure 3b). Interestingly, the abundance of synaptopodin was reduced in MN glomeruli and in c-mip transgenic mice (Figure 3c).