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Immunopathogenic Mechanism and Therapeutic Intervention in an Experimental Murine Model of Membranous Nephropathy

Chia-Chao Wu^{1,2}, Kuo-Cheng Lu³, Jin-Shuen Chen¹,
Yuh-Feng Lin^{1,4} and Huey-Kang Sytwu²

¹*Division of Nephrology, Department of Medicine, Tri-Service General Hospital*

²*Department of Microbiology and Immunology, National Defense Medical Center*

³*Department of Medicine, Cardinal Tien Hospital,
School of Medicine, Fu Jen Catholic University*

⁴*Division of Nephrology, Department of Medicine,
Shuang-Ho Hospital; Taipei Medical University, Taipei,
Taiwan*

1. Introduction

Idiopathic membranous nephropathy (MN), an autoimmune-mediated glomerulonephritis (GN), is one of the most common causes of nephrotic syndrome in adults and may rarely lead to idiopathic nephrotic syndrome in childhood (Cattran, 2001; Chadban & Atkins, 2005; Ponticelli & Passerini, 1990). The clinical course in the majority of patients appears to be indolent and slowly, although some progress into end-stage renal failure. Spontaneous remissions of proteinuria occur in approximately one quarter of patients; approximately half will have stable renal function with or without persistent proteinuria (Rosen, 1971; Wasserstein, 1997). A minority of patients will demonstrate slowly decline in renal function, and a few will have rapidly decline leading to renal failure or death. Approximately 30%–40% of patients with MN develop progressive renal impairment, which results in end-stage renal failure after 10–15 years (Cattran, 2001; Chadban & Atkins, 2005; Honkanen et al., 1992). The etiology in the majority of cases is unknown (idiopathic or primary MN, 75%); MN can also be secondary to or associated with a variety of conditions, including systemic lupus erythematosus and other autoimmune disorders, chronic infections such as with hepatitis B virus, drugs such as penicillamine, and malignant neoplasms (Cattran, 2001; Chadban & Atkins, 2005; Honkanen et al., 1992; Ponticelli & Passerini, 1990; Rosen, 1971; Wasserstein, 1997). The characteristic finding in MN is the presence of *in situ* immune-complex deposition over the subepithelial space which presented with diffuse, discrete, granular glomerular capillary wall staining for immunoglobulins and complement on immunofluorescence and subepithelial electron-dense deposits on electron microscopy. The deposited immune complexes sequentially induce the inflammatory response, complement activation, and oxidative injury, all of which participate in the pathogenesis of MN (Couser

& Abrass, 1988; Couser & Nangaku, 2006; Cunningham & Quigg, 2005; Kerjaschki, 2004; Nangaku et al., 2005; Ronco & Debiec, 2006).

However, the role of adaptive immunity in the mediation of glomerular injury in MN has not yet been fully elucidated (Couser & Abrass, 1988; Couser & Nangaku, 2006; Cunningham & Quigg, 2005; Kerjaschki, 2004; Nangaku et al., 2005; Ronco & Debiec, 2006). The prominent phenomenon of antigen and antibody immune-complex were observed in the process in the Heymann nephritis (HN), one of the most frequently used models of human MN in rat (Heymann et al., 1959; Salant et al., 1989). However, recent experiments with anti-T cell subset monoclonal antibody (mAb) therapies also suggested that T cells are central to the induction and glomerular injury of HN (Quiza et al., 1992). Indeed, the immunopathogenesis of MN, especially the role of T cell (including helper and cytotoxic T cells), is still not clear (Nangaku & Couser, 2005). The treatment of MN is controversial. Controlled trials of corticosteroids with or without cytotoxic or immunosuppressive agents have had variable results. Presently available immunosuppressive therapies are not always effective and often have many persistent side effects (Cattran, 2005; du Buf-Vereijken et al., 2005; Glassock, 2004; Perna et al., 2004; Piccoli et al., 1994; Polenakovik & Grcevska, 1999; Ponticelli et al., 1987; Ponticelli, 1987; Ponticelli & Passerini, 1991). The treatment of patients with MN is still up for debate. The current treatment of this disease remains inadequate and nonspecific. The purpose of this review is to bring together current informations concerning the roles of immunopathogenesis in the development and progression of MN. Specific emphasis is placed on the new murine MN model setup and the kinetics of adaptive immunity during MN. In addition, immuno-modulatory treatments using heme oxygenase-1 (HO-1) induction in the treatment of MN are also reviewed.

2. Immunopathogenic mechanism and therapeutic intervention in experimental murine model of MN

2.1 Experimental animal model of MN

MN is an important cause of nephrotic syndrome and end-stage renal disease in adults. For designing more rationale therapy, it would be useful if we really understood basic pathophysiology of disease. Since only limited information can be obtained from the direct study of diseased humans, animal models are very helpful for us to understand pathogenesis. Most results were obtained from HN. However, limited mouse models for study of human MN was well-characterized although mice offer the advantages of being cheaper, manipulate easily, and suitable for experimental applications.

2.1.1 Heymann Nephritis (HN)

HN is a rat model of autoimmune-mediated glomerulonephritis that is similar in histopathology to human idiopathic MN (Heymann et al., 1959; HEYMANN et al., 1962). In 1959, Heymann *et al.* (Heymann et al., 1959) first described a model of MN in rats induced by immunization with a tissue antigen fraction derived from proximal tubular brush borders (Fx1A) in complete Freund's adjuvant (CFA) (Kerjaschki & Farquhar, 1982; Kerjaschki & Farquhar, 1983). This model, subsequently referred to as HN, the morphology more closely resembled human MN because the deposits were exclusively subepithelial in location (Edgington et al., 1968; Edgington et al., 1967). The "active" HN model took several weeks to develop after immunization. Following, passive HN (PHN)

model by passively transferring heterologous antibodies to the brush border (anti-Fx1A) into normal animals in which antibody deposition induced heavy proteinuria more rapidly within only 3 to 4 days, thus finally enabling studies to be conducted of the mediation of glomerular injury in MN (Dixon et al., 1961; Van Damme et al., 1978; Fleuren et al., 1978). Whether the immune complexes were formed *in situ* or passive trapping of small, soluble immune complexes formed in the circulation is an important issue. Within months, Van Damme *et al.* using *ex vivo* perfusion system, and Couser *et al.* using a physiologically intact isolated perfused kidney system established that the deposits in MN result not from circulating immune complex trapping but from direct, or *in situ*, binding of IgG antibody to native glomerular antigens, presumably expressed on the membrane of podocyte foot processes (Van Damme et al., 1978; Couser et al., 1978). The nature of the glomerular antigen involving in formation of subepithelial deposits in HN was systematically tracked down by Kerjaschki and Farquhar and demonstrated as a combination of megalin and a receptor-associated protein (RAP) in the podocyte membrane (and tubular brush border) (Kerjaschki & Farquhar, 1982; Makker & Singh, 1984; Farquhar et al., 1995). Megalin (gp330) has been identified as a pathogenic antigen in HN, but it has not been found in human or mice glomeruli. Hence, the precise nature of the idiopathic MN-initiating antigen is still unknown. (Ronco & Debiec, 2007b; Ronco & Debiec, 2007a; Ronco & Debiec, 2005).

2.1.2 Murine model of MN induced by cationic bovine serum albumin

The HN is a generalized model with morphological and functional aspects similar to those of human MN. However, it has rarely been applied to the mouse which have advantages of low cost, easily manipulation and the potential application of genetic and monoclonal antibody techniques (Quigg, 2003). Repeated doses of cationic bovine serum albumin (cBSA) are alternative methods to induce MN which had been applied in the dog, cat, rabbit and rat (Wright et al., 1985; Nash et al., 1990; Border et al., 1982; Bass et al., 1990). The purity of the cBSA antigen is an important factor to cause variants of MN. The heterogeneity of the antigenic charge distinctly affects its ability to cause damage: the more cationic the immunogen, the more nephritogenic it is and the greater its tendency to produce a typical MN pattern. Unlike the cBSA preparations previously used only characterized by polyacrylamide gel flat bed electrophoresis and fast protein liquid chromatography (Bass et al., 1990; Wright et al., 1985), which could not exclude the presence of native anionic and slightly cationic BSA, we used pH-dependent binding technique to purify a more homogenous cBSA preparation which made our model disclosing greater consistency (Chen et al., 2004; Wu et al., 2008a; Yang & Langer, 1985). Mice were immunized with 0.2 mg of cBSA emulsified in complete Freund's adjuvant (CFA), two weeks later, these mice received cBSA (13 mg/kg) intravenously three times per week, every other day, for six weeks to induce MN. There was a dose-related effect in the induction of MN and strain specificity because MN could be induced in BALB/c mice, but not in the C57BL/6 strain (Chen et al., 2004; Wu et al., 2008a). All cBSA-induced MN mice developed the characteristically clinical symptoms of proteinuria, hypoalbuminaemia, and hypercholesterolaemia. The MN induction rate did not differ between male and female mice reflecting the equal incidence of MN in men and women. Overt proteinuria appeared in week 4 and reached a plateau at week 8. Serum albumin concentration declined markedly after week 6 in the MN group and reached its nadir at week 8. However, serum cholesterol concentration showed a compensatory increase at

week 6 and was maximal at week 8. The above presentations assemble like nephrotic syndrome in human MN. The blood urea nitrogen and serum creatinine concentrations did not change during MN induction. Urinalysis revealed no haematuria or leukocyturia during the study.

Histological findings revealed characteristically MN morphologic pattern, namely, diffuse thickening of the glomerular basement membrane (GBM) and no significant mesangial proliferation in light microscopy. (Figure 1A) Ultrastructural analysis identified severely irregular thickening of the basement membrane and subepithelial deposits in mice with cBSA-induced MN. (Figure 1D) Positive immunofluorescent staining for IgG was noted by progressively stronger granular fluorescence intensity along the glomerular capillary wall (GCW) with a discrete beaded appearance. Immunofluorescence staining for C3 revealed a similar time course and pattern to that of IgG. (Figure 1B and C) Loss of glomerular anions and the impairment of charge selectivity were noted evidenced by decreased the intensity of colloid iron staining progressively during the course of MN induction. We also found that the Th2 response was predominant: IgG1 concentration was significantly higher than IgG2a concentration. These data indicate that the MN mouse model is associated with a Th2 response. An important issue is to determine whether cBSA-induced MN in mice resembles the *in situ* immune-complex glomerulonephritis seen in humans. Immunofluorescence analysis revealed positive staining for both IgG and C3. Although a strong positive immunofluorescent staining of immune-complex deposition was concomitant with higher serum Ig concentrations, serum CIC concentration did not increase significantly before week 4. These observations suggest the *in situ* immune-complex formation in cBSA-induced MN, although we cannot exclude the possibility that the lower CIC concentration might have been generated in the earlier stages but was deposited rapidly in the kidney. Previous studies using both *in vitro* and *in vivo* approaches have demonstrated that cBSA binds directly to the glomerulus and then forms the *in situ* immune-complex deposition. BSA can induce *in situ* immune-complex formation in the isolated perfused rat kidney (Fleuren et al., 1980). Antigen quantity also seems to be a cofactor influencing MN induction and a threshold exposure to antigen is needed for induction (Breyse et al., 1994)]. Although the mechanisms responsible for this variable course remain unclear, our results suggest that lower antigen exposure might increase the chance of spontaneous remission. We speculate that the antigen source, dose and exposure duration are factors in the pathological and clinical diversity of MN.

We report the development of a new MN mouse model induced by cBSA. Clinically, the animals developed hypoalbuminaemia, hypercholesterolaemia and severe proteinuria. Morphologically, they exhibited diffuse GBM thickening, granular immunofluorescent staining, subepithelial deposits, and a lack of inflammatory cell infiltration and mesangial cells proliferation. Exogenous cBSA antigen had a dose-related influence on disease induction and may have induced *in situ* immune-complex GN. Moreover, this mouse MN model may display Th2 polarization and strain-specific dependence. Extending the MN model to the mouse has the advantages of lower cost, easy manipulation, and the potential benefits of using gene knockout and transgenic mice to investigate the mechanisms of disease initiation and progression. Thus, this model exhibits great similarity to human MN disease in clinical and pathological features over time. This murine model will provide a valuable tool to investigate the pathogenesis of MN and will help in the development of preventive and therapeutic strategies for MN, which is difficult to study in humans.

Fig. 1. Renal histopathology in mice with MN. Histopathology revealed characteristic findings of diffuse basement membrane thickening, as observed in the (A) hematoxylin and eosin staining, (B) positive granular immunofluorescent staining for IgG, (C) positive granular immunofluorescent staining for C3, and (D) subepithelial deposition (asterisk). NC, normal control; MN, membranous nephropathy; G, glomerular basement membrane; L, lumen of capillary; and U, urinary space.

2.2 Immunopathogenesis of cBSA-induced MN

MN has been recognized as an autoimmune-mediated GN and characterized by an *in situ* immune-complex disposition over the subepithelial space which caused physical disruption of the filtration barrier and triggered a cascade of events that contribute to the progression of the disease and result in glomerular injury and proteinuria (Ravnskov, 1998; Ronco & Debiec, 2005). The C-BSA binding to glomerular tuft anion site, then immunoglobulin and complement will be produced and aggregated to GBM and mesangial area. This results in glomerular epithelial cell injury and proteinuria in our cBSA-induced murine MN model. Mediation of glomerular injury in MN has been thought to be essentially leukocyte independent (Salant et al., 1985). Early studies using monoclonal antibodies to T cell subsets indicate that T cells are central to the induction and glomerular injury in HN, a rat model of idiopathic MN (Penny et al., 1997; Penny et al., 1998; Quiza et al., 1992). The T lymphocyte-derived cytokines also regulate the cellular and humoral immune responses to

nephritogenic antigens and modulate inflammatory events (Holdsworth et al., 1999; Kelly et al., 1998). However, the role of adaptive immunity in the mediation of glomerular injury in MN has not yet been fully elucidated.

2.2.1 The role of T cells and their cytokines in MN

It is widely recognized that CD4⁺ T cells can be differentiated into two subsets, Th1 and Th2, according to their cytokine profiles (Abbas et al., 1996; Mosmann & Sad, 1996). Th1 cells produce cytokines such as interleukin (IL) 2, interferon γ IFN- γ , and tumor necrosis factor α TNF- α to promote cell-mediated immunity. Th2 cells producing IL-4, IL-5, IL-10 and IL-13 can suppress Th1 cell activation and contribute to humoral immunity (Mosmann & Coffman, 1989). Th1 and Th2 subsets are regulated reciprocally to maintain a balance, which plays an important role in immune-mediated GN (Tipping & Kitching, 2005). Th1 and Th2 subsets direct diverging effector pathways and lead to different patterns of injury and outcomes in GN. Th1-predominant responses are strongly associated with proliferative and crescentic forms of GN while Th2 responses are associated with membranous patterns of injury (Schena, 1999; Ring & Lakkis, 1998). To our knowledge, the diversity of Th1/Th2 in MN is still controversial. Studies involving patients with idiopathic MN showed a consistently negative response for delay type hypersensitivity (DTH) effectors, an increase in IL-4 production by peripheral T helper cells; and a predominance of IgG4 (Th2-type subclass) as well as complement deposition in glomeruli; suggesting a Th2 response (Oliveira, 1998; Kuroki et al., 2005; Masutani et al., 2004; Hirayama et al., 2002; Doi et al., 1984; Doi et al., 1991; Haas, 1994; Imai et al., 1997; Iskandar et al., 1992; Roberts et al., 1983). The above findings favor Th2 response. However, Penny *et al* found that the progressive development of infiltrates of activated T cells—principally Th1 and cytotoxic effector cells—as well as macrophages identified within glomeruli of Lewis rats with HN, coincides with the development of proteinuria (Penny et al., 1998). Early studies suggested that classical T cell effector responses are involved in HN, including passive transfer of HN to tolerant rats by lymphoid cells but not serum, and lymphoid cells in culture (Heymann et al., 1962; Hsieh et al., 2000). Furthermore, it has also been found that permanent CD8⁺ T cell depletion both early and late in the course of disease prevents proteinuria in active Heymann Nephritis. It may indicate that CD8⁺ cytotoxic T cells are essential to the mediation of glomerular injury in HN and may be relevant to the pathogenesis of MN (Penny et al., 1998). In addition,, Spicer et al found that IL-4 administration prior to the onset of proteinuria, rather than the early rIL-4 treatment prevents the development of proteinuria in active Heymann Nephritis by inhibition of Tc1 cells (Spicer et al., 2001). All these results indicate the importance of CD8⁺ T cell in mediating the final effector phase of glomerular injury in HN (Penny et al., 1998; Spicer et al. 2001) and suggest that Th1 cells participate in the glomerular injury and proteinuria of MN (Penny et al., 1997). The conclusion of the diversity of Th1/Th2 in MN are not consistent.

2.2.2 The kinetics of adaptive immunity of cBSA-induced in MN

The kinetic distribution of different lymphocyte subsets, as well as the association between clinical manifestations and the complicated course of adaptive immune responses during MN is not well understood. To directly investigate immunoregulatory process and immunopathological mechanisms in cBSA-induced MN, we used TH1/TH2 double transgenic mouse (T1/T2 TG mice) which provides the best *in vivo* model to study the differentiation of helper T cell subsets during the Th process was used (Wu et al., 2007).

T1/T2 TG mice, originally in BALB/c background, bear two transgenes expressing two distinct cell surface markers, one is human Thy1 protein (human CD90) under the murine IFN γ promoter control, the other is murine Thy1.1 protein (murine CD90.1) under the murine IL-4 promoter control, designated as TH1/TH2 transgenes respectively (Hsieh et al., 2000; Hung et al., 2005).

T1/T2 TG MN mice showed overt proteinuria, hypoalbuminemia, and hypercholesterolemia characteristically. Renal histopathology revealed typical morphology of MN as glomerular basement membrane thickening, IgG granular deposition and subepithelial deposits seen in H & E, IF and EM sections. There were no significant differences between the MN mice of the BALB/c strain and the T1/T2 TG mice. The numbers of splenic lymphocytes increased progressively. The absolute numbers of CD4 $^{+}$ and CD8 $^{+}$ T cells showed reciprocal increased as total splenocytes despite relative consistent proportion. A progressive increase in the proportion and absolute numbers of CD19 $^{+}$ B cells in the MN mice from week 2; this peaked at week 4 and persistent high throughout the MN process were also observed. Two weeks after immunization, the MN mice had a significant increase in splenic Th2 cells. This indicates an extended Th2 response at this point. We also noticed a trend of increase in the percentage of Th1 cells and most of the remaining Th1 $^{+}$ cells is CD8 $^{+}$ T cells. The percentage of DX5 $^{+}$ cells, a marker for NK cell, in the MN mice did not differ from that in the controls and there were very few cells expressing hCD90 and DX5 markers simultaneously which suggested that most of the Th1 $^{+}$ cells were T lymphocytes, but not IFN- γ producing NK cells. The expression levels of Th1 and Th2 cells among peripheral mononuclear blood cells from MN mice showed similar results. Whether what happening with the T cells in the spleen is also happening in the kidneys, we checked the expression of Th1/Th2 reporter in the kidneys by using quantitative RT-PCR at various time points. The Th1 reporter/Th2 reporter status of kidney revealed parallel changes with those in the spleen. Taken together, these results imply a progressive Th2 prone process of adaptive immunity during experimental MN. We also performed immunohistochemical staining for hCD90, mCD90.1 and CD4 in the kidneys to identify and localize the T-helper cells. The numbers of CD4 $^{+}$ cells were very scanty in NC kidney and the numbers were increased in MN kidney. In contrast to hCD90, there were no mCD90.1 $^{+}$ cells noted in NC kidney, but prominent mCD90.1 $^{+}$ cells were noted in MN mice. The prominent and parallel expression between the mCD90.1 $^{+}$ and CD4 $^{+}$ cells may imply the Th2 polarization in renal T cell during MN.

The Th1 and Th2 subsets can be differentiated according to the production of individual cytokines, Th1 regulates Ig switching to the IgG2a subtype, whereas Th2 enhances IgG1 secretion. We found that the titers of anti-cBSA immunoglobulins – mostly IgG1 – increased from week 2 and peaked at week 4. Serum level of immunoglobulins at week 6 and week 8 were lower than those at week 4. These findings may imply the development of tolerance and a Th2-predominant response. We further performed the immunofluorescent staining to investigate whether the isotype of antibodies deposited in the kidney parallels to the findings in sera, and the result revealed a prominent IgG1 antibody deposition in the kidney rather than IgG2a. A progressive increase in the proportion and absolute numbers of CD19 $^{+}$ B cells in the MN mice during the early phase of MN combined with the production and deposition of IgG1 predominant anti-cBSA immunoglobulin in the serum and kidneys indicate that the initial immune response primarily involves the humoral-mediated mechanism. Recent studies have demonstrated that blocking the CD20-positive B cells by rituximab, and thereby, inhibiting B-cell differentiation and immunoglobulin secretion, can reduce proteinuria and prevent disease progression in patients with idiopathic MN. This

further confirms the pathogenic role of B cells in MN (Cohen et al., 2005; Remuzzi et al., 2002; Ruggenenti et al., 2003). In our previous study, the cBSA-induced MN can be induced in Th2-prone BALB/c mice, but not in Th1-prone C57BL/6 mice (Chen et al., 2004). In this study, we further showed that the Th2 CD4⁺ response, and not the Th1 CD4⁺ response, significantly correlated with the progression of MN. All the results confirmed the relevant role of B cells participating in the process of MN.

We further investigated the secretion of IFN- γ and IL-4 from splenocytes to confirm whether cBSA is a specific antigen in this MN model by using antigen-specific stimulation test stimulated with or without cBSA. As compared to NC mice, levels of IL-4 were increased significantly in MN mice and cBSA restimulation may further enhance its secretion. We identified a significant increase in IL-4 producing cells in the MN group without specific antigen re-stimulation. However, Th1 cells were only slightly amplified. Interestingly, lymphocyte subsets from the MN group re-stimulated with specific antigens displayed a greater capacity to secrete IFN- γ and IL-4, especially among the IFN- γ -producing CD8⁺ T cells and IL-4-producing CD19⁺ B cells. Nevertheless, CD8⁺ T cells contributed more to the augmented production of IFN- γ than did CD4⁺ T cells; moreover, CD19⁺ B cells also demonstrated greater IL-4 production than the CD4⁺ T cells. Quantitative real-time PCR of renal cortex and splenocytes demonstrated a consistent change with slightly increased expression of proinflammatory (TNF- α and IL-6) and Th1 cytokines (IL-2, IFN- γ) and extremely high expression of Th2 (IL-4 and IL-10) cytokines. Finally, we found that the Th2 CD4⁺ response increased as the MN progression and showed that the number of Th2 cell not the Th1 cell significant correlated with serum cholesterol and proteinuria.

In the MN mice, production via cBSA re-stimulation and subsequent staining of intracellular cytokines from cultured splenocytes revealed a significant increase in IL-4-producing CD4⁺ T cells, but only a slight increase in IFN- γ -producing CD4⁺ T cells. This provides further evidence confirming the maintenance of Th2 cells post cBSA induction. Furthermore, data from intracellular cytokines post Ag-specific re-stimulation suggests that IFN- γ is secreted predominantly by CD8⁺ T cells, while IL-4 is secreted by either CD4⁺ T cells or B cells. These results may imply that CD4⁺ T cells preserve their potential Th2 capacity after MN and that CD8⁺ T cells function more effectively than CD4⁺ T cells in the production of IFN- γ following re-exposure to antigens. In addition to assisting in antibody production, the T helper cell subsets also affect and direct cellular immune mechanisms in GN (Holdsworth et al. 1999). Interestingly, we found a latter increase in Th1 expression. The activation of CD8⁺ T cells appears to be much slower than that of the CD4⁺ T cell-dependent B cell response to cBSA. Previous studies have demonstrated that persistent depletion of CD8⁺ T cell both early and late in the course of disease prevented the development of proteinuria. In addition, it was also found that IL-4 administration prior to the onset of proteinuria, rather than the early rIL-4 treatment prevented proteinuria in HN, indicating the importance of CD8⁺ T cell in mediating the final effector phase of glomerular injury in HN (Penny et al., 1998; Spicer et al., 2001). This increase in Th1 cells may play a role in the counter-regulation of the Th2 response and implies an association between the characteristics of the disease process and the kinetics of the Th1/Th2 responses during MN. Taken together, these findings support that the functional dichotomy between the Th1 and Th2 lymphocyte subsets play a regulatory role in the disease and both the humoral and cell mediated immune responses may participate in the pathogenesis of MN.

T1/T2 TG mice still has advantages of stable and stronger signal expression, a wider time window for detection, lack of requirement of restimulation or permeabilization, and

possible isolation of viable cells of given Th phenotypes. Thus, these T1/T2 TG mice are useful in providing a feasible and direct *in vivo* monitoring system for dissecting the changes in proportions of pathogenic T cells during the pathogenic or therapeutic processes using flow cytometry with surface immunofluorescent staining (Hsieh et al., 2000; Hung et al., 2005; Sung et al., 2004).

A progressive increase in Th2 cells were observed in splenocytes as well as in peripheral blood and kidney cells. In addition, the Th2 prone IgG1 immunoglobulin subclass was also noted in the serum and kidneys. Antigen-specific re-stimulation testing of cytokine production and intracellular cytokine staining revealed an IL-4 prominent immune response. Cytokine-related gene expression in the kidneys and splenocytes demonstrated enhancement of pro-inflammatory cytokines as well as Th1/Th2 cytokines. In conclusion, our data demonstrates that both peripheral and renal immune responses are strongly polarized toward the Th2 type immune response in the process of cBSA-induced MN. The T1/T2 double transgenic mice could provide an available model to dissect the complex kinetic changes of adaptive immunity in GN and promises a potential strategy for the development of immunotherapeutic strategies against MN in the future.

2.3 Immunomodulatory treatment using HO-1 induction for cBSA-induced MN

The central pathogenesis participating in MN involves the formation of subepithelial immune deposits and the subsequent production of glomerular injury through complement-dependent processes, oxidative stress, and inflammatory cytokines, resulting in the development of massive proteinuria (Nangaku et al. 2005). However, the appropriate treatment of patients with MN is still open to debate. Recently, HO-1 has been noted to have biological effects for protective and therapeutic use (Ryter et al., 2006; Kirkby & Adin, 2006). On the other hand, presently available immunosuppressive therapies are not always effective and often have many persistent side effects (Glassock, 2004). Therefore, whether HO-1 induction could be applied in MN treatment was discussed.

2.3.1 Heme oxygenase-1

Heme oxygenase (HO) is the rate-limiting enzyme that degrades heme into carbon monoxide (CO), ferritin, and biliverdin (Tracz et al., 2007a). Three distinct HO enzymes have been identified: HO-1, HO-2, and HO-3. The HO-1 identified as an enzyme in microsomes and was described as a new member of the cytochrome p450 family and later found to be a rapidly and transiently inducible mono-oxygenase. The inducible HO-1 is expressed in response to various stimuli, such as hydrogen peroxide, heat, heavy metal ions, hyperoxide, endotoxin, and inflammatory cytokines, whereas another isoenzyme of HO, HO-2, is constitutively expressed and abundant in testes, brain, liver and vasculature. HO-1 has been shown to have cytoprotective properties, as well as anti-inflammatory, anti-oxidant, anti-apoptotic, and possible immunomodulatory functions (Ryter et al., 2006; Kirkby & Adin, 2006; Nath, 2006). Using chemical inducers or genes, HO-1 has been shown to be expressed in various diseases, including respiratory diseases, cardiovascular diseases, renal disease, ocular diseases, liver injury and organ transplantation in animal models (Abraham & Kappas, 2005; Agarwal & Nick, 2000).

2.3.2 HO-1 induction for MN treatment

MN mice receiving HO-1 inducer, cobalt protoporphyrin (CoPP), treatment revealed a marked attenuation of proteinuria, hypoalbuminaemia and hypercholesterolaemia (Wu et

al., 2008b). However, HO-1 inhibitor, tin protoporphyrin (SnPP), treatment did not have such therapeutic effects. The pathological severities of the kidney from MN mice receiving CoPP (MN-CoPP) were milder than those from the MN and MN receiving SnPP mice (MN-SnPP). All these three experimental groups showed positive immunofluorescent staining for IgG, with a discrete beaded appearance, along the glomerular capillary wall, but the immunofluorescence intensity in the MN-CoPP mice was lower than that in the MN-SnPP and MN mice. Immunofluorescent staining for C3 also presented as intense granular fluorescence along the glomerular capillary wall, with a similar pattern to that of IgG. Next, we checked the serum levels of anti-cBSA antibodies in mice to investigate whether the induction of HO-1 modulates the production of the immunoglobulins, causing the subsequent decrease in the immunofluorescence intensity of IgG and C3 during the course of experimental MN. Significantly elevated the levels of serum anti-cBSA antibodies were observed in the MN, MN-CoPP, and MN-SnPP groups compared with those of the control groups. Compared with control MN mice, CoPP treatment inhibited and SnPP treatment enhanced the production of immunoglobulins during MN course.

Oxidative stress plays an important pathogenic role in MN. We further assessed whether CoPP or SnPP treatment modulates the production of oxidative stress systemically in the serum and locally in kidneys. We checked the lipid peroxidation products, thiobarbituric acid reactive substances (TBARS), as markers of oxidative stress. The serum TBARS levels in MN mice were significantly higher than those in NC mice. CoPP treatment effectively attenuated the levels of TBARS to a level similar to that observed in NC mice. However, SnPP, which inhibits HO-1, did not reduce the level of oxidative stress, as indicated by the higher levels of serum TBARS observed in MN mice treated with SnPP. TBARS in the kidney displayed a similar pattern to that observed in serum. These findings suggest that HO-1 reciprocally affects both local and systemic oxidative stress. We also analyze *in situ* superoxide anion radical production by using DHE assay in fresh-frozen sections of renal tissue to more specifically and locally detect the ROS production in the kidney which revealed similar results as TBARS. Anti-apoptosis effect is another major cytoprotective function of HO-1. We then checked whether HO-1 induction alleviates cell apoptosis in MN. There were nearly undetectable TUNEL-positive cells, as an index of cell apoptosis, in normal mouse kidneys. However, increased numbers of apoptotic cells were detected in the glomeruli and surrounding tubules of MN mice. Compared with MN mice, little apoptosis was observed after MN mice were treated with CoPP, as indicated by a decrease in the number of TUNEL-positive nuclei. The administration of SnPP failed to reduce the apoptosis in kidney cells. Therefore, the anti-apoptotic effect induced by CoPP may also contribute to the therapeutic effect of HO-1 in MN mice.

In addition to its anti-oxidative and anti-apoptotic properties, HO-1 also displays well documented anti-inflammatory activity. We examined the mRNA expression of inflammation-associated cytokines in the renal cortex to answer the question of whether the induction of HO-1 modulates the inflammatory state. The expression of pro-inflammatory cytokines (IL-1 β and TNF- α), Th1 cytokines (IFN- γ and IL-2), Th2 cytokines (IL-4 and IL-10), the fibrogenic cytokine transforming growth factor (TGF) β , and HO-1 were checked. Quantitative real-time PCR of the renal cortex demonstrated a consistent change in MN, with an increased expression of pro-inflammatory, Th1 cytokines and Th2 cytokines. CoPP treatment dramatically induced HO-1 expression in the kidneys, decreased the level of pro-inflammatory cytokines (IL-1 β and TNF- α), and extremely increased the level of anti-

inflammatory cytokine IL-10. SnPP also decreased the pro-inflammatory cytokines, but did not induce the anti-inflammatory cytokine IL-10.

Previous studies demonstrated that oxygen radical scavengers can dramatically reduce proteinuria in HN (Neale et al., 1994). In our study, MN mice treated with CoPP showed a dramatic reduction in the generation of highly reactive compounds of lipid peroxidation products, TBARS, and in proteinuria. These results indicate that oxidative stress may play a pathogenic role in damaging the glomerular filtration barrier, and could be causally related to proteinuria in experimental MN (Nangaku et al., 2005). The decrease in TUNEL-positive kidney cells after CoPP treatment observed in our study also demonstrates the anti-apoptotic properties of HO-1 (Ryter et al., 2006; Nath, 2006). The metabolic derivatives of heme produced by HO-1 (CO and biliverdin) have powerful anti-apoptotic and anti-oxidative properties. Whether the cytoprotective capacity of HO-1 in experimental MN is exerted via these byproducts requires further investigation. However, there was no prominent infiltration of inflammatory cells in our murine MN model. Therefore, we speculate that the resident kidney cells contribute to the induction of HO-1. Renal vascular and tubular structures express HO-1, particularly in response to injurious conditions (Abraham & Kappas, 2005). Glomeruli have also been reported to express HO-1 in the human kidney in various renal diseases (Morimoto et al., 2001). It seems that site-specific expression along the nephron or in the interstitium occurs due to the proximity of the stimulus. In our study, it was difficult to clarify whether the protective effect of HO-1 was mediated through systemic or local action, because both effects coexisted when the mice received CoPP treatment. Previous studies using either the exogenous administration of HO-1 by gene transfer specifically expressed in the kidney or chemical induction or transgenic mice, all demonstrated the therapeutic effects of HO-1 in different disease models (Kirkby & Adin, 2006; Agarwal & Nick, 2000). Therefore, we assume that both the systemic immunomodulatory effect, which decreases the production of immunoglobulins and subsequently or directly reduces inflammation, complement activation, oxidative stress, and apoptosis, and the local effect in the MN glomeruli contribute to the therapeutic effect of HO-1.

The HO-1 induction caused the effective attenuation of proteinuria via multiple mechanisms, including immunomodulatory, anti-oxidative, and anti-apoptotic effects. (Figure 2) CoPP-induced HO-1 suppressed the synthesis of pro-inflammatory cytokines such as IL-1 β , IFN- γ and TNF- α . Furthermore, it stimulated the production of the anti-inflammatory cytokine, IL-10. It has been postulated that the degradation products of heme and its metabolic derivatives (CO in particular) might contribute to the anti-inflammatory functions of HO-1. The CO-mediated anti-inflammatory effect caused by the increasing production of IL-10 and the inhibition of TNF- α and IL-1 β has been reported to be mediated through interactions with the MAPK signaling pathways (Ryter et al., 2006). In contrast, IL-10 mediates the immunosuppressive effect by an HO-1-dependent pathway (Lee & Chau, 2002). CoPP treatment also significantly reduced the production of serum anti-cBSA antibodies and glomerular immunodeposits in experimental MN mice. It has been demonstrated that the immunomodulatory effect of HO-1 is associated with regulatory T-cell *Foxp3* expression and T-cell proliferation via IL-2 (Brusko et al., 2005; Pae et al., 2004). However, the role of HO-1 in the reduction of immunoglobulin production remained unclear. The direct action of induced HO-1 against systemic inflammation, the concomitant decrease in the production of immunoglobulins, together with the subsequent decrease in immunodeposition, and other factors such as complement activation and inflammation, may all contribute to the attenuation of proteinuria. In our study, both CoPP and SnPP

decreased the pro-inflammatory cytokines, but only CoPP increased the production of the anti-inflammatory cytokine IL-10. Apart from the regulation of pro-inflammatory and anti-inflammatory cytokines, CoPP also decreased the level of immunoglobulin production, complement activation, and oxidative stress, which have been proposed to be major pathogenic factors in MN. This may partially explain why only CoPP, and not SnPP, exerted a therapeutic effect in alleviating experimental MN in our study. In contrast, the deficiency of HO-1 impairs renal hemodynamics and exaggerates systemic inflammatory response in mice (Tracz et al., 2007c; Tracz et al., 2007b).

Fig. 2. Cytoprotection by HO-1 in experimental MN. HO-1 induction therapy decreased immunoglobulin production and subsequently or directly attenuated inflammation, complement activation, oxidative stress, and apoptosis in MN glomeruli, all of which contributed to the amelioration of disease severity in MN mice. End, glomerular endothelial cell; Epi, glomerular epithelial cell; GBM, glomerular basement membrane; MC, mesangial cell.

The development of ideal therapeutic agents that can effectively and specifically blunt the pathogenic pathway in MN is an important issue. This is the first study to demonstrate that the endogenous induction of HO-1 significantly ameliorates proteinuria and the severity of pathology in MN mice. The induction of HO-1 suppressed the production and deposition of immune complexes in the kidney. Both systemic and local oxidative stresses were reduced in the sera and kidneys of CoPP-treated MN mice compared with those of MN mice or SnPP-treated MN mice. Apoptosis in the kidney cells was also reduced after treatment with CoPP. Conversely, decreased pro-inflammatory cytokine expression and increased anti-

inflammatory cytokine expression were observed in the kidneys after treatment with CoPP. The efficient administration of CoPP once weekly, which can significantly block the key inflammatory, oxidative, and immunomodulatory pathogenesis pathways of MN, make HO-1-inducing therapeutic regimens a plausible new option for future therapeutic interventions in MN. However, there are still no drugs available that have been specifically developed to induce HO-1 and can be applied to human clinical use. Although the therapeutic effects of HO-1 have been demonstrated in several diseases in animal models, including our experimental MN model, whether it can be applied to humans requires further investigation.

Our results suggest that HO-1 induction therapy ameliorates experimental MN via multiple pathways, including anti-oxidative, anti-apoptotic, and immunomodulatory effects. HO-1-inducing regimens will probably be considered a new therapeutic intervention for MN in the future.

3. Conclusion

Murine model of MN induced by cBSA exhibited great similarity in clinical and pathological features to human MN disease. This murine model will provide a valuable means to investigate the pathogenesis of MN and will help in the development of preventive and therapeutic strategies for MN. Both peripheral and renal immune responses are strongly polarized toward the Th2 type immune response in the process of cBSA-induced MN. The T1/T2 double transgenic mice could provide an available model to dissect the complex kinetic changes of adaptive immunity in GN and promises a potential strategy for the development of immunotherapeutic strategies against MN in the future. Finally, HO-1 induction therapy ameliorates experimental MN via multiple pathways, including anti-oxidative, anti-apoptotic, and immunomodulatory effects. HO-1-inducing regimens will probably be considered a new therapeutic intervention for MN in the future.

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Phone: +86-21-62489820
Fax: +86-21-62489821

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