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A Protective Role of Mer Receptor Tyrosine Kinase in Nephrotoxic Serum-induced Nephritis

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Abstract

The Mer receptor tyrosine kinase is strongly expressed in the glomerulus. We wondered if this molecule might modify immune-mediated glomerular disease through its functions as a receptor for apoptotic cells and immunoregulatory molecule. Mer-knockout (KO) mice showed decreased survival rate and greatly increased proteinuria and serum urea levels compared to wild type (WT) mice by day 3 after injection of NTS. Their glomeruli were hyperplastic and later became necrotic. While in the glomerulus of WT mice, a significant increase of Mer expression was observed. Apoptotic bodies were evident in NTS-treated Mer-KO kidneys, but not in normal controls. NTS-treated Mer-KO mice had massive neutrophil infiltration and inflammatory cytokine expression. Mer thus has a critical role in attenuating renal inflammation, both as a receptor for apoptotic cells and as a molecule that down regulates inflammation.

Keywords

MerTK; glomerulonephritis; apoptotic clearance; cytokine regulation

Introduction

Nephrotoxic serum (NTS)-mediated kidney disease is a well-studied mouse model of glomerulonephritis (GN) and has proven to be a valuable model providing insights into the mechanism of lupus nephritis [1]. The severity of injury correlates directly with the dose of antibody injected. Histological changes induced by inflammatory injury exhibit apoptosis related subtly degrees of abnormalities. Disordered clearance of apoptotic cells is likely to account for the presence of cell fragments in severe lupus GN [2]. PMNs play a particular key role in the early pathogenesis of NTS-nephritis [3]. Infiltrated PMNs are activated by deposited immune complexes, which results in glomerular injury [4]. The expression of cytokines and chemokines is also known to play a critical role in both heterologous and autologous stages of NTS-nephritis. Monocyte chemoattractant protein-1 (MCP-1), a CC-chemokine, is mainly

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released by activated monocyte/macrophages, and attracts leukocytes and other mediators to sites of inflammation [5;6]; TNF acts in the recruitment of inflammatory cells and subsequent development of proliferative glomerulonephritis. Mice deficient in TNF α / β are protected from NTS glomerulonephritis [7].

The Mer receptor tyrosine kinase (MerTK) belongs to the Tyro-3 subfamily of receptor tyrosine kinases, which includes Tyro-3, Axl, and Mer. Receptors from this subfamily share common ligand: growth-arrest specific protein 6 (Gas6) [8;9]. Though still debatable, protein S, a Gas6 related vitamin-K dependent anticoagulation factor, has also been shown as a ligand at least for Tyro-3 and MerTK [10;11;12]. Axl/Gas6 signaling was shown to promote the development of inflammatory renal disease partially through increased proliferation rate of mesangial cells [13]. Gas6 knock out mice showed reduced pathological changes in experimental nephritis [14]. Though, both Axl and Gas6 are barely detectable in normal kidneys of mice and humans, the expression level of both genes was reported increase in disease phase [14;15].

The importance of Mer in the clearance of apoptotic cells in the immune system together with its function in attenuating immune responses through modulation of cytokine production has drawn much attention to this molecule in the field of autoimmunity. Mer deficient mice develop a lupus-like autoimmune syndrome, which most likely results from Mer-mediated inflammatory response with skewed cytokine production and impaired engulfment of apoptotic cells [8;16;17]. Mer receptor signaling induces an inhibitory pathway in macrophages regulating TNF- α production through blockage of NF- κ B cascade [16;18]. The expression pattern of Mer has been reported mostly within the monocytic cell lineage with imbalanced tissue distribution. The kidney has been shown to have highest amount of message RNA compared to a panel of other tissues [19]. We have detected high levels of Mer protein in the kidney by Western blot and routinely use kidney lysates as a positive control (unpublished data). In our current study, we have localized the expression of renal Mer more precisely to the glomerulus, and found that it was upregulated during experimental glomerulonephritis. Strikingly, the Mer-KO mice were much more susceptible to NTS-nephritis than WT. Within 3 days of NTS injection, we observed severe renal damage in Mer-KO mice yet not in WT controls. We further explored the mechanism of protective role of Mer in the development of NTS-mediated nephritis and found that early onset renal damage in Mer-KO mice was associated with increased inflammatory cytokines, excessive apoptotic cells, and massive infiltration of neutrophils.

Materials and Methods

Materials and mice

Biotin conjugated anti-mouse Mer antibody was purchased from R&D Systems (Minneapolis, MN). FITC Anti-sheep IgG was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-mouse IgG and Gr-1 antibodies were from BD Pharmingen (San Jose, CA). Glomerular cell subtype specific antibodies, anti-nephrin, anti- α SMA (smooth muscle actin), anti-WT-1, and the second FITC conjugated donkey anti-rabbit were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The WT control mice C57BL/6J (B6) were originally purchased from Jackson Laboratories (Bar Harbor, ME). Mer-KO mice were generated and backcrossed to B6 mice as previously described [16]. Mice were subsequently bred and maintained in our mouse colony at Temple University. All of the experimental procedures performed on these animals were conducted according to the guidelines of the Institutional Animal Care and Use Committee.

Immunofluorescent staining

Mice kidneys were embedded in OCT medium and snap-frozen in acetone cooled with dry ice. Sections (4 μ m) were air-dried for 10 minutes, followed by 30 minutes incubation with 1%

BSA in PBS. Sample sections were then blocked with 3% BSA in PBS. Mer expression was detected by incubation with biotin-labeled anti-Mer at 4°C and visualized with PE-streptavidin. All other antibodies were incubated with tissue sections for 2 hrs at 4°C. Slides were mounted in Anti-fading Aqueous Mounting Medium (Biomedica Corp., Foster City, CA), and images were acquired using an Olympus BX60 fluorescence microscope equipped with camera (Center Valley, PA).

Induction of nephrotoxic nephritis and histological analysis

Sheep nephrotoxic sera were prepared as previously described [20]. In brief, mice glomeruli were isolated from normal B6 mice by differential sieving and used to hyperimmunize sheep. The nephrotoxic serum was heat-inactivated and absorbed with an excess amount of murine blood cells. Nephrotoxic sera were aliquoted and frozen at -80°C for later use. Mice were given sheep NTS as a single dose intravenously (7.5 ml of serum per kg mouse weight). Mice were then followed daily with measurement of proteinuria (Uristix, Bayer Corporation, Elkhart, IN) and serum urea level using Urea Nitrogen Direct kit (Stanbio Laboratory, Boerne, TX). At day 3, kidneys were removed, either fixed in 10% buffered formalin (H&E) or frozen in OCT medium (Immunofluorescence). 4 µm sections from paraffin embedded samples were stained with H&E and periodic acid-Schiff (PAS). Pathological evaluation by light microscopy was done in a blinded manner by M. B.

Antibody deposition analysis

Four µm frozen sections were air-dried and blocked with 3% BSA, followed by FITC-conjugated anti-sheep IgG and FITC anti-mouse IgG antibodies staining, respectively. For quantitative immunofluorescence, sections were examined in a blind manner at 40 × magnification. The mean intensity of 20 glomeruli for each sample was analyzed for evaluation.

TUNEL assay

Cell apoptosis was analyzed by TUNEL assay using In Situ Cell Death Detection Kit (Fluorescein, Roche Diagnostics, Indianapolis, IN). Briefly, sections were pretreated with proteinase K solution, followed by 1 h incubation with TUNEL reaction mixture (Label solution: fluorescein labeled dUTP and enzyme solution: terminal deoxynucleotidyl transferase (TdT) enzyme) at 37°C in the dark. Apoptotic cells were identified as TUNEL-positive cells showing green fluorescence. Apoptosis was assessed by examining more than three sections per animal.

Real-time PCR

Total RNA was isolated using RNeasy (Qiagen Inc., Valencia, CA) from whole kidney lysates. The first strand cDNA was synthesized from 1 µg of total RNA using reverse transcriptase and 1 µmol/L of oligo-dT primer (RETROscript, Ambion Inc., Austin, TX). Each cDNA sample was amplified by using specific primers against MCP-1 (sense: 5'-aggctcctgtcatgcttctgg-3' and anti-sense: 5'-acagtccgagtcacactagtcca-3'), IL-1β (sense: 5'-gggtgtgtgacgttcattaga-3' and anti-sense: 5'-catggagaatatcactgttggtga-3'), IL-6 (sense: 5'-tcaattccagaaaccgctatga-3' and anti-sense: 5'-gaagtagggaaggccgtggt-3'), TNF-α (sense: 5'-cgtggaactggcagaagagg-3' and anti-sense: 5'-ctgccacaagcaggaatgag-3'). The house keeping gene β-actin (sense: 5'-gacggccaggtcactat-3' and anti-sense: 5'-acatctgctggaaggtggac-3') was assayed in parallel, as an internal control. Real-time PCR was performed in an Eppendorf realplex² Real-Time PCR system (Eppendorf, Westbury, NY) using DNA Master SYBRgreen dye (Applied Biosystems Inc. Foster City, CA). The C_T of each test message was first normalized using the C_T for β-actin, assayed in the same sample. Fold change was then calculated using the relative C method: Fold change = 2^(normalized C_T in stimulated sample - normalized C_T in resting sample).

Statistical analysis

Statistical differences in each group were calculated with Student's t test. $P < 0.05$ was considered significant. Data are expressed as means $\pm$ SD.

Results

Mer is expressed in glomeruli

The expression of Mer in mouse kidney has been reported both at the mRNA [19] and protein levels [21]. We sought to define the expression pattern of Mer within the kidney. Mouse kidneys were snap frozen and sectioned at 4 μ m thickness. Mer expression was detected using immunofluorescent staining by incubating with biotin conjugated goat anti-mouse Mer antibody and visualized with PE-streptavidin. As shown in Figure 1, Mer was expressed in the glomeruli of kidney from WT B6 mice, but not Mer-KO mice. The lack of non-specific staining was also confirmed with isotype control antibody from WT (Figure 1A) and Mer-KO (not shown) mice. Further differential staining using antibodies recognizing antigens on cell subtype in the glomeruli revealed an expression pattern of Mer on mesangial cells and glomerular endothelial cells but not podocytes (Figure 1B).

Mer-KO mice showed increased mortality, higher proteinuria, and serum urea levels

To determine the effects of Mer in the development and progression of chronic renal disease, nephrotoxic nephritis was induced in both Mer-KO and WT mice with 7.5 ml of sheep nephrotoxic serum per kg mouse body weight. Under these conditions, 60% of Mer-KO mice died between day 4 and day 15, while all wild type mice survived until day 16 (Figure 2A). Urine samples from Mer-KO mice appeared grossly bloody shortly before death. In Mer-KO mice with nephritis, excretion of urinary albumin and serum levels of urea were increased as early as the second day after injection (Figure 2B and C). Proteinuria reached a plateau on day 4. In contrast, significant less proteinuria was observed in WT mice at each comparable time point (Figure 2C). WT mice developed slowly progressive renal disease, with gradually increasing proteinuria and blood urea levels (Figure 2B and 2C).

Mer-KO mice developed severe glomerular injury with increased PMN infiltration

At day 3, approximately 20% of the glomeruli from Mer-KO mice showed dramatically occluded and distended capillaries filled with PAS-positive fibrinous material, which was not observed in WT mice. In addition, proteinaceous tubular casts were more frequently observed in Mer-KO mice compared with WT mice (Figure 3). No significant inflammation was observed in the renal interstitium from either Mer-KO or WT mice at day 3.

Immune complex deposition in Mer-KO mice kidney

It is possible that the enhanced glomerular damage in the Mer-KO mice was due to increased deposition of nephritogenic immune complexes. In our initial analysis, however, the apparent amount of linear glomerular capillary deposition of sheep anti-mouse GBM antibodies was identically strong in both WT and Mer-KO mice (Figure 4A). The subsequent deposition of immune complexes indicative of the endogenous response to sheep IgG as detected with an anti-mouse IgG antibody was virtually no difference between Mer-KO and WT mice (Figure 4B). These data indicate that Mer deficiency does not affect the binding/deposition of sheep anti-mouse GBM, or the production and deposition of mouse anti-sheep IgG.

Enhanced Mer expression and PMN infiltration in the glomeruli from NTS-nephritis mice

The observation of early onset kidney failure in Mer-KO mice indicates a protective role of Mer in the development of NTS-nephritis. This is consistent with our demonstration of Mer protein expression in the normal glomerulus (Figure 1). It was particularly impressive, then,

that we could demonstrate greatly enhanced Mer expression, confined to the glomeruli of WT mice three days after challenge with nephrotoxic serum (Figure 5). At this time point, Mer-KO mice did not show staining with the anti-Mer reagent, even though they had greatly advanced renal inflammation. Thus, the WT kidney up-regulates glomerular Mer expression in response to a potentially damaging challenge with nephrotoxic serum, and the Mer protein indeed appears to suppress tissue damage at the early stage (3 days).

PMNs are the first responders in NTS-mediated renal inflammation and play a major role in early renal damage upon inflammation. To investigate whether Mer deletion enhances the infiltration of PMNs into kidney, we stained kidney sections from Mer-KO and WT mice with PE labeled anti-granulocyte antibody, Gr-1. Figure 6 showed massive infiltration of PMNs in the kidneys of Mer-KO mice but not of WT mice at day 3 post immunization.

Nephritic Mer-KO mice showed elevated MCP-1 and TNF

Cytokines and chemokines play an important role in the course of NTS-nephritis. Cytokine profile changes are highly dependent on the type of nephrotoxic serum, animal strains studied, the phase of disease development, and the amount of serum administered. Mer has been shown to modulate immune response through down regulation of inflammatory cytokines. Among all cytokines [22], IL-1 and IL-6 are known to be important in the heterologous phase; while TNF and MCP-1 are essential for disease development in the autologous phase. In our model, we detected a significantly high level of MCP-1 in kidneys from Mer-KO mice compared to WT mice at both stages after serum injection (Figure 7). This increase in expression correlated with the increased infiltration of PMNs (Figure 6) in the kidney at early stages. Mer-KO mice showed significantly high TNF- α and IL-6 production at the autologous phase but not the heterologous phase, while IL-1 production was significantly upregulated at heterologous stage in Mer-KO mice comparing to WT mice (Figure 7).

Mer-KO mice with nephritis showed increased amounts of apoptotic bodies

Mer serves as a major receptor on professional phagocytes such as macrophages in Gas6-mediated phagocytosis [23]. It has also been shown to promote proliferation and to inhibit apoptosis. Mer functions to insure rapid and efficient clearance of apoptotic cells for the prevention of secondary necrosis and the release of pro-inflammatory cytokines, which can injure surrounding tissues. To determine the amount of apoptotic cell bodies in the kidney during NTS-nephritis, we detected apoptosis at the single cell level by labeling DNA strand breaks with TUNEL assay. As shown in Figure 8, apoptotic cells accumulated in kidney samples from Mer-KO mice 3 days after nephritis induction. TUNEL-positive cells were not only limited to the tubules but were also observed to some extent in the glomeruli in Mer-KO mice, but not in the WT mice (Figure 8). No obvious apoptotic bodies could be identified in naïve Mer-KO mice (data not shown). Kidney samples from normal WT mice served as negative controls (no treatment) and positive controls (DNase I treated), respectively.

Discussion

The prominent expression of Mer in the kidney has raised the question of its role in this organ, especially in light of the susceptibility of the kidney to antibody-mediated injury. The present study demonstrates, for the first time, that Mer expression is on mesangial and endothelial cells within the glomerulus. The partial overlap of nephrin with Mer expression could be argued by the interdigitating processes between podocytes (epithelium) and capillaries (endothelium), the latter has the maximum expression of Mer. Mer-KO mice kidneys are far more susceptible to antibody-mediated damage in the absence of a functional Mer molecule. These findings extend previous work ascribing a protective role to Mer in other kinds of inflammation [8;9; 16;17], and contrast with the previously reported role of Axl in accelerating renal disease

[13;24]. Thus, Mer and Axl, two receptor kinases and members of the same subfamily, seem to serve opposing roles in modulating or promoting glomerular inflammation. The contrasting roles of Mer and Axl in vivo may be dependent on their relative expression (much greater for Mer in the basal state) and in the availability of ligands. Protein S, a negative regulator of coagulation, is abundant in unstimulated plasma and may serve to bind to the large amounts of Mer in healthy kidney [10;11;24]. When kidneys become inflamed, both Gas6 and Axl expression is increased, opening up the possibility that involvement of the Axl pathway may serve to exacerbate inflammation once it begins. Further experiments will be required to clarify the apparently complex relationship between Axl and Mer in renal pathology.

A key function of Mer is to help in clearing apoptotic cells [17;23;25;26]. Our data confirm that Mer serves this function in the kidney, where many cells undergo apoptosis, especially during nephritis. It is possible that Mer's protective role is partially through the clearance of apoptotic cells, so that they cannot serve to stimulate further autoimmunity through progression to necrosis and provision of autoimmunogenic nuclear antigens. Through this mechanism, almost any renal insult causing apoptosis might have the potential to stimulate autoimmunity if nuclear debris is allowed to accumulate. Mer may serve to protect against secondary formation of anti-nuclear antibodies when kidneys undergo injury of any kind.

Increased infiltration with leukocytes is now recognized in virtually all forms of glomerular injury. The adherence and subsequent transmigration of circulating monocytes into glomerular mesangium occurs early in the development of renal diseases. Our studies reinforce previous observations that myeloid cell infiltration in the glomeruli and the tubulointerstitium is essential to disease in the NTS-nephritis model [1]. In previous studies [16] and in present experiments (Figure 7), elevated amount of TNF- α was observed in inflamed organ lacking Mer. TNF- α has been approved by many in vitro and in vivo studies, as a fundamental proinflammatory cytokine involved in the pathogenesis of glomerular injury [1;7;27;28]. Mesangial cells activated by TNF- α transcribe MCP-1 mRNA in a dose- and time-dependent manner [29]. MCP-1 has been thought to play an important role in the recruitment and accumulation of monocytes within the glomerulus. Accumulated observations suggest that MCP-1 accounts for nearly all of the monocyte chemotactic activity [30;31;32]. Matsukawa A., et al demonstrated that MCP-1 serves as an indirect mediator to attract neutrophils via the production of leukotriene B₄ (LTB₄) [33]. The striking effect of Mer in preventing neutrophil infiltration appears to be due to a dampening of MCP-1 expression, and adds this cytokine to the list of cytokines whose production can be regulated by Mer ligation. Together with previous data [1;34], IL-1 and IL-6 production also appeared to be down regulated by Mer, contributing to its protective role.

The increased expression of Mer in injured kidneys in these experiments deserves comment. Mer expression has also been observed to increase on B cells in chronic GVH disease. In the present model, increased Mer expression may serve as an additional regulatory mechanism to protect renal tissue against self-reactive humoral immunity, and therapeutics aimed at enhancing Mer expression might be rational agents to reduce ongoing renal inflammation.

The present studies reinforce the view that Mer and its ligands are key participants in autoimmune and inflammatory processes. Better understanding of how capillary rich organs such as the kidney are protected through Mer may lead to new approaches to modulate immune-mediated injury.

Acknowledgments

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Abbreviations

MerTK	Mer receptor tyrosine kinase
Gas6	growth arrest-specific protein 6
NTS	nephrotoxic serum
GN	glomerulonephritis
B6	C57BL/6J

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Figure 1. Mer is expressed in mouse glomeruli

Mouse kidney sections (4 μ m) were preblocked with 3% BSA in 1 $\times$ PBS, followed by biotin conjugated anti-Mer antibody incubation. A. Expression of Mer in kidney was visualized by incubating with streptavidin-PE. Biotin labeled goat IgG was used as negative control (Magnification: $\times$ 100 upper panel; $\times$ 400, lower panel.). B. Co-localization of Mer (red) with mesangial cells (α SMA, green), glomerular endothelial cells (WT-1, green), or podocytes (nephryn, green) was evaluated in glomeruli of WT B6 mice. Images were acquired using an Olympus BX60 fluorescence microscope equipped with camera (Center Valley, PA). Magnification: $\times$ 400.

Figure 2. Mer-KO mice showed severe renal injury

Nephritis was induced as indicated in methods. A. Survival curves of WT mice (solid triangle) and Mer-KO mice (solid circle). B. Serum urea levels of WT mice (open bar) and Mer-KO mice (solid bar). C. Proteinuria levels of WT mice (open square) and Mer-KO mice (solid rhombus). Data are expressed as mean $\pm$ SD. * $p < 0.05$. ** $p < 0.01$.

Figure 3. Histological changes of kidneys from WT and Mer-KO mice

Mice were euthanized at day 3 and kidneys were fixed in 10% formalin. Kidneys were then sectioned at 4 μ m thickness, followed by H&E (A) and PAS staining (B). Slides were evaluated by M. Birkenbach in a blinded manner. Control groups were Mer-KO mice injected with non-specific sheep serum (control serum). Data are representative of three individual experiments. Magnification: $\times 100$.

Figure 4. Glomerular antibody deposition in Mer-KO mice kidney

Three mice from the WT and Mer-KO group were sacrificed at day 3. Mice kidneys were snap-frozen in dry ice cooled acetone. Kidney sections (4 μ m) were blocked and stained for FITC anti-sheep IgG (A) and FITC anti-mouse IgG (B). Data shown here are representatives of three individual experiments. Magnification $\times$ 400.

Figure 5. Mer expression was upregulated upon nephritis induction

Mer expression in the kidney was evaluated in the kidney three days after nephritis induction comparing to the untreated mice. Kidney sections were processed as in figure 1A. Nephritic Mer-KO mice were used as negative control. A. Representative data. B. Quantitative data (MFI, mean fluorescent intensity) from three experiments. Magnification, $\times 100$.

Figure 6. PMN infiltration into Mer-KO mice kidney

Kidney sections were stained with PE conjugated anti-mouse Gr-1 monoclonal antibody. At day 3, kidney samples from three individual experimental nephritis were examined under fluorescent microscope. Representative data were shown. Magnification, $\times 100$.

Figure 7. Measurement of TNF- α , IL-1, IL-6, and MCP-1 gene expression from WT and Mer-KO mice

Three kidney samples were homogenized for total mRNA preparation at day 3 and day 8 after nephrotoxic serum injection. mRNA levels of the 4 molecules were assayed by real-time PCR. The fold changes are with respect to mRNA levels on day 0 and was calculated after normalized against β -actin message.

Figure 8. Increased apoptotic bodies in Mer-KO mice kidney

Three days after nephrotoxic serum injection, kidney samples from WT and Mer-KO mice (top panel) were snap-frozen and subjected to TUNEL staining as described in the materials and methods. Controls (bottom panel) were from naïve WT animals with (positive) or without (negative) DNase I treatment. Magnification, $\times 400$.