p38 MAPK phosphorylation and NF-κB activation in human crescentic glomerulonephritis

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Abstract

Background. p38 mitogen-activated protein kinase (p38 MAPK) followed by the activation of NF-κB participates in the intracellular signal transduction and production of cytokines and chemokines. The pathophysiological roles of p38 MAPK and NF-κB in human glomerulonephritis, however, remain to be investigated.

Methods. We investigated the phosphorylated p38 MAPK (p-p38 MAPK) and activated NF-κB immuno-histochemically in the kidneys of 34 patients with crescentic glomerulonephritis and 26 control patients with thin basement membrane disease and minimal change nephrotic syndrome. We also explored the co-localization of p-p38 MAPK with CCR5, the signal of which leads to p38 MAPK activation. Furthermore, urinary levels of MIP-1α, the cognate ligand for CCR5, were determined by enzyme-linked immunosorbent assay.

Results. p-p38 MAPK-positive cells and activated NF-κB-positive cells were mainly detected in crescentic lesions, tubular epithelial cells, and interstitial mononuclear infiltrates. The number of p-p38 MAPK-positive cells in patients with crescentic glomerulonephritis was higher than that in control patients. The number of p-p38 MAPK-positive cells in glomeruli was well correlated with the percentage of cellular crescents, the number of CD68-positive cells, and urinary MIP-1α levels. In addition, the number of activated NF-κB-positive cells was well correlated with the number of p-p38 MAPK-positive cells in glomeruli. Dual staining revealed that most of CCR5-positive cells were positive for p-p38 MAPK. Finally, p-p38 MAPK-positive cells and activated NF-κB-positive cells decreased during glucocorticoid therapy-induced convalescence.

Conclusions. We conclude that the phosphorylation of p38 MAPK associated with the activation of NF-κB may be involved in the upregulation of intrarenal MIP-1α and the utilization of CCR5 signalling, which may result in human crescentic glomerulonephritis.

Keywords: CCR5; chemokine receptor; crescentic glomerulonephritis; MIP-1α; NF-κB; p38 MAPK

Introduction

Mitogen-activated protein kinases (MAPKs) are major intracellular signal transduction factors, through which signals from environmental stimuli are transmitted to the nucleus [1]. Thus far, at least three distinct groups of MAPKs have been identified; extracellular signal-regulated kinases (ERK), c-Jun NH2-terminal kinase/stress-activated protein kinase (JNK/SAPK), and p38 MAPK [1]. p38 MAPK is phosphorylated in response to hyperosmolar and oxidative stresses, and inflammatory cytokines including interleukin (IL)-1β and tumor necrosis factor (TNF)-α [1,2]. Furthermore, phosphorylated p38 MAPK (p-p38 MAPK) contributes to the activation of nuclear transcription factors including nuclear factor kappa B (NF-κB), which regulates the gene expression of various cytokines, chemokines, and adhesion molecules [3,4]. Recently, p38 MAPK is thought to be involved in the pathogenesis of experimental rheumatoid arthritis model [5] and human Sjögren’s syndrome [6]. In addition, p38 MAPK takes part in an experimental renal disease [7]. To date, however, the studies addressing the role
of p38 MAPK activation in human renal diseases are sparse.

Crescentic glomerulonephritis is a prominent feature in rapidly progressive glomerulonephritis (RPGN), resulting in end-stage renal failure [8]. Macrophage inflammatory protein-1α (MIP-1α), a member of C-C chemokine family, is secreted by mononuclear cells, neutrophils, and renal resident cells and has a potential to recruit and activate macrophages (Mφ) and T cells [9]. It is reported that MIP-1α is involved in the pathogenesis of experimental and human crescentic glomerulonephritis, especially in cellular crescents [10,11]. In addition, CCR5, the cognate receptor for MIP-1α, is detected in crescentic lesions and the signal transduction of MIP-1α via CCR5 may be involved in the pathogenesis of crescentic glomerulonephritis [11,12]. Recent studies revealed that the phosphorylation of p38 MAPK is involved in the signal transduction through CCR5 in vitro [13]. Furthermore, the production of MIP-1α is regulated by the activation of NF-κB [14] and the phosphorylation of p38 MAPK [15]. However, the roles of p38 MAPK phosphorylation in NF-κB activation and MIP-1α production remain unexplored in the pathogenesis of human crescentic glomerulonephritis.

We studied here the involvement of p38 MAPK phosphorylation in terms of NF-κB activation and the upregulation of MIP-1α in human crescentic glomerulonephritis, especially glomerular lesions. We also examined the impact of glucocorticoid therapy on p38 MAPK phosphorylation and NF-κB activation in human crescentic glomerulonephritis.

**Subjects and methods**

**Subjects**

Sixty patients (31 males and 29 females; median age 56.4 years) with primary or secondary glomerular diseases were evaluated in this study (Table 1). Thirty-four patients (18 males and 16 females; median age 61.1 years) had crescentic glomerulonephritis with more than 50% of the total crescents (cellular, fibrocellular, and fibrous) of all glomeruli showing RPGN clinically. Sixteen patients with minimal change nephrotic syndrome (MCNS; seven males and nine females; median age 54.7 years) and 10 patients with thin basement membrane disease (TBMD; six males and four females; median age 51.5 years) served as disease controls. The patients in this study were chosen consecutively from January 1971 to March 2000 at Kanazawa University Hospital or its affiliated hospitals. All diagnoses were verified by renal biopsy. Urinary tract infections were ruled out in all cases by means of bacterial cultures and/or the microscopic findings, because urinary tract infection is associated with increased urinary MIP-1α levels (data not shown). Whenever possible patients did not receive immunosuppressive agents before sample collection. All patients in a clinically active state were treated with glucocorticoids including methylprednisolone pulse therapy (500–1000 mg/day, 3 days) during this study. Specimens from second biopsies were obtained from 12 patients of crescentic glomerulonephritis after glucocorticoid therapy to evaluate disease activities in kidneys. All renal biopsies were performed with the informed consent of the patients.

**Pathological studies**

Seventy-two kidney specimens were obtained by the first and second renal biopsies. Two observers, with no knowledge of the clinical course of the patients, examined the renal tissue under a light microscope to establish the diagnosis by standard pathological methods. The crescentic formation in all glomeruli obtained was counted and the percentage of crescents was expressed per glomerulus.

**Immunohistochemical studies**

The presence of p38 MAPK, p-p38 MAPK, and NF-κB active form was demonstrated immunohistochemically on formalin-fixed, paraffin-embedded tissue specimens treated with catalysed signal amplification system (DAKO, Glostrup, Denmark) using the indirect avidin-biotinylated peroxidase complex method. As primary antibodies, we used a murine monoclonal anti-human p38 MAPK antibody (10 μg/ml; Santa Cruz Biotech. Inc., Santa Cruz, CA) and a murine monoclonal anti-human p-p38 MAPK antibody (10 μg/ml; Santa Cruz Biotech. Inc.). In addition, to detect NF-κB active form in the diseased kidneys, we used a murine monoclonal anti-human NF-κB (p65 subunit) antibody (10 μg/ml; Chemicon, Tamecula, CA), which detects only the p65 subunit released from its inhibitor, inhibitory κB (IκB), and which thus specifically recognizes activated p65 in diseased kidneys [16]. Normal mouse IgG1, which had been absorbed with both human liver extracts and immunoglobulin, was used as a negative control. In addition, blocking peptides for p38 MAPK and p-p38 MAPK (Santa Cruz Biotech. Inc.) were used for the absorption tests as negative controls. As a positive control, synovial tissues from patients with rheumatoid arthritis were used. Furthermore, to determine the localization of CCR5 or CD68 in renal tissue, we performed immunohistochemical analyses on formalin-fixed, paraffin-embedded sections by the indirect avidin-biotinylated alkaline phosphatase complex method with a specific murine monoclonal anti-human CCR5 antibody (10 μg/ml; clone 2D7; Pharmingen, San Diego, CA) or
murine monoclonal anti-human CD68 antibody (10 μg/ml; clone KP1; DAKO) as described previously [11]. In addition, a dual immunostaining technique was used to determine the characterization of p-p38 MAPK-positive cells as described before [12]. Briefly, CCR5 was detected by the indirect avidin-biotinylated alkaline phosphatase method as described above. Then, the slides were blocked by 0.1% sodium azide and 3% H2O2 in methanol for 15 min. After this process, p-p38 MAPK was detected using the indirect avidin-biotinylated peroxidase complex method as described above. In this dual immunostaining, the colour of p-p38 MAPK-positive cells is brown, and that of CCR5-positive cells is red. Glomerular p38 MAPK-, p-p38 MAPK-, NF-κB active form-, CCR5- and CD68-positive cells were expressed as the number of positive cells per glomerulus. Two independent observers also examined the immunohistochemical findings without a prior knowledge of chemokine levels and the clinical courses.

Urinary MIP-1α measurements

Spontaneously voided midstream urine catches were collected on the morning of renal biopsy. Urinary MIP-1α levels were determined by an enzyme-linked immunosorbent assay (ELISA) as described previously [11]. The recovery rate was confirmed to be more than 95% up to 3 ng/ml in these ELISA systems. All assays were performed in duplicate. The detection limits of this ELISA system were 66 pg/ml for human MIP-1α. Urinary MIP-1α levels were standardized by the amount of creatinine in the urine.

Detection of anti-neutrophilic cytoplasmic antibody by ELISA

Anti-neutrophilic cytoplasmic antibody (ANCA) was detected by ELISA using microtitre plates coated with myeloperoxidase extracts for P-ANCA or proteinase-3 for C-ANCA (SRL Co., Tokyo, Japan) [17].

Statistics

Statistical significance was analysed using paired or unpaired Student’s t-test, ANOVA test, Spearman’s and Pearson’s correlation coefficient for the analyses of nonparametric and parametric data. P < 0.05 was considered to be statistically significant.

Results

Immunohistochemical detection of p38 MAPK and p-p38 MAPK in renal tissues

To determine the renal localization of p38 MAPK and p-p38 MAPK, renal tissues from 34 patients with crescentic glomerulonephritis, 16 patients with MCNS, and 10 patients with TBMD were examined by immunohistochemical analyses. In patients with crescentic glomerulonephritis, p-p38 MAPK was observed in the nuclei of crescentic lesions, tubular epithelial cells, and mononuclear infiltrates in interstitium (Figure 1A). The number of p-p38 MAPK-positive cells in glomeruli in patients with crescentic glomerulonephritis (4.2 ± 0.4/glomerulus) was higher than those in patients with MCNS (0.9 ± 0.1/glomerulus) and TBMD (0.7 ± 0.3/glomerulus) (P < 0.01, Figure 1B). In contrast, p38 MAPK was detected mainly in the cytoplasm of glomerular cells and tubular epithelial cells in all patients (Figure 2A–C). Moreover, the number of p38 MAPK-positive cells in the glomeruli did not differ among these three groups (data not shown). In synovial tissues from patients with rheumatoid arthritis, we detected positive staining for p38 MAPK and p-p38 MAPK, respectively (data not shown). The staining was specific for p38 MAPK and p-p38 MAPK, because neither mouse IgG1 nor antibodies absorbed with excess blocking peptides stained positively (data not shown).

![Fig. 1.](image-url) (A) Immunohistochemical examination of p-p38 MAPK in a representative patient with crescentic glomerulonephritis. p-p38 MAPK-positive cells were detected mainly in crescentic lesions (arrows) and interstitial mononuclear infiltrates (arrowhead). (B) The number of p-p38 MAPK-positive cells in glomeruli is shown in patients with crescentic glomerulonephritis, MCNS and TBMD. Bars indicate mean ± SEM. *P < 0.01.
Co-localization of p-p38 MAPK and CCR5

To determine the characterization of p-p38 MAPK-positive cells, we explored the localization of p-p38 MAPK with respect to CCR5 by a dual staining. In patients with crescentic glomerulonephritis, most of the CCR5-positive cells in the glomeruli were positive for p-p38 MAPK staining (Figure 3).

Correlation of the number of p-p38 MAPK-positive cells with the pathological findings

The number of glomerular p-p38 MAPK-positive cells was well correlated with the percentage of cellular crescents ($r = 0.551$, $P < 0.01$, $n = 34$, Table 2). In contrast, it did not give a significant correlation with the percentage of fibrous or fibrocellular crescents (Table 2). In addition, CD68-positive cells were detected in crescentic lesions and interstitial infiltrates. The number of glomerular p-p38 MAPK-positive cells was well correlated with the number of CD68-positive cells in the glomeruli ($r = 0.587$, $P < 0.01$, $n = 34$, Table 2).

Correlation of the number of p-p38 MAPK-positive cells with urinary MIP-1α levels

Urinary levels of MIP-1α were elevated before glucocorticoid therapy in patients with crescentic glomerulonephritis (4.1 ± 2.3 pg/mg·creatinine). In contrast, we did not detect urinary MIP-1α in patients with MCNS or TBMD. In addition, the number of p-p38 MAPK-positive cells in the glomeruli was significantly correlated with urinary MIP-1α levels ($r = 0.689$, $P < 0.01$, $n = 34$, Table 2).

Immunohistochemical detection of activated NF-κB in renal tissues

In patients with crescentic glomerulonephritis, activated NF-κB was detected in the nuclei of necrotizing and crescentic lesions, tubular epithelial cells, and mononuclear infiltrates in the interstitium (Figure 4A).

The number of activated NF-κB-positive cells in the glomeruli in patients with crescentic glomerulonephritis (5.7 ± 0.4/glomerulus) was higher than those in patients with MCNS (0.5 ± 0.4/glomerulus) and TBMD (0.8 ± 0.4/glomerulus) ($P < 0.01$, Figure 4B). Moreover, the number of p-p38 MAPK-positive cells in the glomeruli was significantly correlated with the number of activated NF-κB-positive cells in the glomeruli ($r = 0.639$, $P < 0.01$, $n = 34$, Table 2).
Activated NF-κB-positive cells were detected in synovial tissues of patients with rheumatoid arthritis, while they were not detected using normal mouse IgG in negative control (data not shown). Therefore, the staining was specific for activated NF-κB.

**Correlation of the number of glomerular activated NF-κB-positive cells with the pathological findings and urinary MIP-1α levels**

The number of glomerular activated NF-κB-positive cells was well correlated with the number of CD68-positive cells in glomeruli ($r=0.671$, $P<0.05$, $n=34$) and urinary MIP-1α levels ($r=0.676$, $P<0.01$, $n=34$). In contrast, the number of glomerular activated NF-κB-positive cells did not correlate with the percentage of any types of crescents (data not shown).

**Effects of glucocorticoid therapy on the phosphorylation of p38 MAPK, NF-κB activation and urinary MIP-1α levels in glomeruli**

Twelve of 34 patients treated with glucocorticoid therapy received the second renal biopsy. The number of glomerular p-p38 MAPK-positive cells was significantly decreased during convalescence induced by glucocorticoid therapy including methylprednisolone pulse therapy ($4.7 \pm 0.6$ vs $2.1 \pm 0.4$, $P<0.01$, Figure 5A). Similarly, the number of activated NF-κB-positive cells in glomeruli was decreased

Fig. 4. (A) Detection of activated NF-κB-positive cells in glomeruli. Activated NF-κB-positive cells were detected mainly in crescentic lesions (arrows) and interstitial mononuclear infiltrates (arrowhead) in a representative patient with crescentic glomerulonephritis. (B) The number of activated NF-κB-positive cells in glomeruli is shown in patients with crescentic glomerulonephritis, MCNS, and TBMD. Bars indicate mean ± SEM. *$P<0.01$.

Fig. 5. Alteration of the number of p-p38 MAPK-positive cells in glomeruli (A) and activated NF-κB-positive cells in glomeruli (B) following glucocorticoid therapy in patients with crescentic glomerulonephritis. Bars indicate mean ± SEM. *$P<0.01$. 
after glucocorticoid therapy (4.4 ± 0.3 vs 2.2 ± 0.5, P < 0.01, Figure 5B).

**Discussion**

In the present study, we investigated whether the p38 MAPK signalling system participates in the pathogenesis of human crescentic glomerulonephritis. The phosphorylation of p38 MAPK and activation of NF-κB were detected in the glomeruli, especially crescentic lesions, in patients with crescentic glomerulonephritis. In addition, the number of p-p38 MAPK-positive cells was well correlated with the percentages of cellular crescents, the number of activated NF-κB-positive cells, and urinary levels of MIP-1α. Furthermore, most of the CCR5-positive cells were positive for p-p38 MAPK staining. Taken together, this study suggests that the phosphorylation of p38 MAPK contributes to human crescentic glomerulonephritis via the activation of NF-κB and the subsequent upregulation of MIP-1α. Thus, p38 MAPK may be a novel therapeutic target for human crescentic glomerulonephritis.

The data addressing the pathophysiological roles of MAPK activation in human diseases is sparse. p38 MAPK phosphorylation is reported to be involved in human Sjögren’s syndrome [6] and Alzheimer’s disease [18] thus far. In contrast to these diseases, the involvement of MAPK in human renal diseases remains to be investigated, although the activity of phospholipase A2, an effector of activated ERK, was increased in the urine of patients with mesangial proliferative glomerulonephritis [19]. Recent studies revealed that the activation of ERK, JNK, and p38 MAPK in renal tissue participates in the glomerular injury of some experimental renal diseases, such as anti-glomerular basement membrane glomerulonephritis and mesangial proliferative glomerulonephritis [20,21]. We firstly described here the involvement of p38 MAPK especially in crescent formation of human crescentic glomerulonephritis. In addition, we reported previously that FR167653, a specific inhibitor of p38 MAPK activation, ameliorated rat crescentic glomerulonephritis [22]. Therefore, collecting these findings, p38 MAPK activation involved in crescentic glomerulonephritis may be the therapeutic target in future.

Activated Mφ causes renal tissue injury by secreting inflammatory mediators, such as cytokines and chemokines, releasing lysosomal enzymes and generating superoxide anions [23]. In addition, the interactions of activated Mφ with renal resident cells, including glomerular epithelial cells, may play a crucial role in the formation of crescents via the upregulation of adhesion molecules and matrix formation [23]. Recently, we reported that MIP-1α, which has a potential to recruit and activate Mφ, plays an important role in the pathogenesis of human crescentic glomerulonephritis, especially cellular crescent formation [11]. The present study showed that the number of glomerular p-p38 MAPK-positive cells was well correlated with the number of glomerular activated NF-κB- and CD68-positive cells and the levels of urinary MIP-1α. In addition, this study showed that the number of glomerular activated NF-κB-positive cells was well correlated with the number of glomerular CD68-positive cells and the levels of urinary MIP-1α.

Recent studies revealed that the expression of the MIP-1α gene is regulated by the phosphorylation of p38 MAPK and activation of NF-κB [14,15]. Therefore, the results suggest that the phosphorylation of p38 MAPK closely associated with the activation of NF-κB participates in human crescentic glomerulonephritis, via MIP-1α production.

Immunohistochemical analyses confirmed that most of CCR5-positive cells in the glomeruli were p-p38 MAPK-positive cells. CCR5 is a member of G-protein-coupled receptor superfamily, and binds the MIP-1α, MIP-1β, and RANTES [24]. Furthermore, p38 MAPK is required for CCR5-mediated signal transduction pathway through the activation of the related adhesion focal tyrosine kinase, which is a member of the focal adhesion kinase family [13]. In addition, recent studies suggest that two different subsets of T helper cells (Th1 and Th2) produce distinct immune responses associated with distinct patterns of cytokine secretion and chemokine receptor expression [25]. Crescentic glomerulonephritis is thought to be mediated by Th1-predominant nephritogenic immune responses [25]. CCR5 has been reported to be expressed on human Th1 cells and Mφ [26]. Recently, we reported that glomerular CCR5-positive cells participate in glomerular lesions including extracapillary lesions [12]. Taken together, this study suggests that CCR5-positive activated intraglomerular Mφ and T cells may utilize p38 MAPK signalling cascades, culminating in cell-mediated crescentic glomerulonephritis.

To date, five isoforms of p38 MAPK have been identified; p38α, p38β, p38β2, p38γ, and p38δ [2]. Recent studies revealed that inflammatory cells (neutrophils, Mφ and CD4+ T cells) synthesize the p38α protein predominantly; therefore, the phosphorylation of p38α is involved mainly in inflammatory diseases [2]. The anti-human p-p38 MAPK antibody we used in this study recognized both p-p38α and p-p38β; therefore, further studies will be required to identify which isoform of p38 MAPK is specifically involved in human crescentic glomerulonephritis.

In this study, the number of glomerular p-p38 MAPK-positive cells and activated NF-κB-positive cells significantly decreased during convalescence induced by glucocorticoid therapy including methylprednisolone pulse therapy. In addition, we reported previously that urinary MIP-1α levels were decreased by glucocorticoid therapy [11]. The efficacy of methylprednisolone-pulse therapy has been established for the treatment of RPGN based on improved prognosis [27]. Thus far, the precise actions of glucocorticoid therapy on renal injury remain unclear. However, recent studies reported that glucocorticoid inactivates...
NF-κB by inducing inhibitory protein called IκB, which has a potential to inactivate NF-κB by masking the nuclear localization signal of NF-κB [28]. Therefore, it would be reasonable to speculate that glucocorticoid inactivates NF-κB, in turn, reduced the production of MIP-1α. These may halt lethal downstream events, resulting in clinical convalescence of human crescentic glomerulonephritis.

In conclusion, the pathogenesis of human crescentic glomerulonephritis is closely related to the phosphorylation of p38 MAPK, which may activate NF-κB in the diseased kidneys. In turn, these events upregulate the production and signalling cascades of MIP-1α including p38 MAPK and CCR5, thereby, perpetuates human crescentic glomerulonephritis. Taken together, the p38 MAPK-dependent amplification feedback loop may be a therapeutic target for human crescentic glomerulonephritis.

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