

# TAK-603, an anti-inflammatory compound, reduces crescentic glomerulonephritis and preserves renal function in WKY rats

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**TAK-503 ameliorates crescentic glomerulonephritis in WKY rats**

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Short title: Impacts of Th1 inhibitor on crescentic glomerulonephritis

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## ***Introduction***

Crescentic glomerulonephropathy is characterized clinically by rapid deterioration of renal function, and histologically by mononuclear cell infiltration of the glomeruli, glomerular cell proliferation, crescent formation and eventually glomerulosclerosis. A number of studies have attempted to control cellular pathways that contribute to autoimmune responses and the subsequent inflammatory cascade that leads to prigrressive autoimmune disease(1). The mechanisms controlling progression of glomerulonephritis are poorly understood. An imbalance in the T helper 1 (Th1)/Th2 ratio is thought to be indicative of pathogenesis. In past studies crescentic nephritis is thought to be Th1 predomoinance(1,2).

In Wistar-Kyoto (WKY) rats, a very small dose of nephrotoxic serum induces severe proliferative and necrotizing glomerulonephritis with crescentic formation, resembling human crescentic glomerulonephritis. Renal damage eventually leads to glemerulonephritis and interstitial fibrosis(3). WKY rats are prone to Th1 immune responses, and useful model to analyse the mechanism of crescentic glomerulonephritis.

Antirheumatoid arthritis drug TAK-603, (ethyl 4-(3,4-dimethoxyphenyl)-6,7-dimethoxy-2-(1,2,4-triazol-1-ylmethyl)-quinoline-3-carboxylate), reduces Th1 cytokines, such as IFN- $\gamma$ , IL-2, IL-12(4). In past studies, this drug controls the progression of synovial injury and arthritis in adjuvant arthritis rats(5), therapeutic effect on graft versus host disease(4), and rat skin allograft survival(6). However, the mechanism how this drug affects is still unknown.

Therefore we hypothesized that TAK-603 supresses Th1 dominant T cells, and ameliorates crescentic glomerulonephritis. To achieve this goal, we evaluate crescentic glomerulonephritis induced by WKY rats and rats treated with TAK-603. We report here that suppression of Th1 cytokines represents a beneficial therapeutic approach for crescentic glomerulonephritis.

## ***Material and methods***

### *Animals*

Male WKY rats, ages 8 to 10 weeks were purchased from Charles River Japan Inc (Atsugi, Japan). All procedures used in animal experiments complied with standards in the *Guidelines for the Care and Use of Laboratory Animals in Takara-machi Campus of Kanazawa University*.

### *Preparation of Antirat Glomerular Basement Membrane Antibodies*

Rat glomerular basement membrane (GBM) was prepared using the method of Krakower and Greenspon(7). Preparation of antirat GBM antibodies was described previously(3). Specificity was confirmed by in vitro indirect immunofluorescence using fluorescein isothiocyanate (FITC)-conjugated antirabbit immunoglobulin G (IgG; no.38236; Organon Teknika Corp, Durham, NC) on frozen sections of normal Wistar rat kidneys.

### *Experimental Design*

Forty-eight male WKY rats were injected intravenously with 0.1mL of nephrotoxic serum day 0. WKY rats were divided into three groups, and experimental design was described in Figure 1. TAK-603 (50mg/kg body weight), dissolved in methylcellulose, was orally administrated, starting at the time of induction of glomerulonephritis. Twelve of 48 rats were administered daily for 6 days. Nephritic rats were killed at day 6 (6 rats) or at day 56 (6 rats), and blood samples were collected. Similarly twelve rats were administered at day 0 and killed at day 6 (6 rats) and day 56 (6rats), and twelve rats were administered from day 3 to 5, and killed at day6 (6 rats) and day 56 (6 rats). Twelve rats were treated daily vehicle (methylcellulose) alone for 6 days and used as negative controls.

### *Histopathologic Studies*

A portion of renal tissue was fixed in 10% buffered formalin, followed by embedding in paraffin and staining with hematoxylin and eosin, as well as periodic acid-Schiff reagent. To avoid variation in the shape of glomeruli, glomeruli of the same diameter from a vascular pole were chosen. Crescentic formation was counted from more than 30 glomeruli for each rat and expressed as a percentage of positive to total glomeruli. Cell

populations infiltrating glomeruli were counted in 10 randomly chosen glomeruli, and the result was expressed as total cell numbers per glomerus.

#### *Immunohistology*

To analyse cell populations infiltrating glomeruli, cryostat sections were stained with either a mouse monoclonal antibody against ED-1 (BMA Biomedicals Ltd.Augst,Switzerland) for monocyte and macrophages, CD4, and CD8 (BMA Biomedicals Ltd.Switzerland) for CD4+ lymphocytes, followed by FITC-conjugated rabbit antimouse IgG (Cappel, West Chester, PA, USA). Positive cells were counted in at least 10 randomly chosen glomeruli and the result was expressed as number of positive cells per glomerular cross-section.

#### *Electron microscopy*

Renal tissue was fixed in 2.5% glutalaldehyde and 4% osmic acid, followed by embedding in Epok812 (Oken , Tokyo) and staining with uranyl acetate. This specimen was observed with electron microscopy (Hitachi, Tokyo, Japan).

#### *Determination of Urinary Protein Concentration, Creatinine, IFN- $\gamma$ and IL-4*

Urinary protein concentrations were determined before immunization, days 0, 6 and 56 at the time rats were killed. Urinary protein excretion was expressed as the total amount excreted in a 24-hour period. Urinary creatinine level was measured using an automated analyzer (Hitachi, Tokyo, Japan) according to the manufacture's instructions. Urinary IFN-  $\gamma$  and IL-4 were measured by an enzyme-linked immunosorbent assay (R&D systems, Mineapolis, MN, USA). Urinary Protein Concentration, IFN-  $\gamma$  and IL-4 were collected by urinary creatinine.

#### *RNA isolation and RT-PCR*

Total cellular RNA was isolated from the cortices, and analyses were performed using reverse transcriptase polymerase chain reaction (RT-PCR), as previously described(8). Reverse transcription for 6  $\mu$  g total RNA, from 6 rats in each group(1  $\mu$  g RNA per a rat), was performed using a RT-PCR kit(Perkin Elmer, Foster City, CA, USA). The complementary DNA product(1  $\mu$  g) was amplified by PCR. The primer of rat IL-12p35 was purchased from Biosource International(amplified fragment of 555bp)(9), and the

other primers used for amplification are shown in Table 1. Ten microliters of PCR products were analyzed using 2% agarose gel electrophoresis, and stained with ethidium bromide. The housekeeping gene GAPDH was used for PCR controls.

#### *Statistical analyses*

Mean and SE were calculated on all parameters determined in this study. Statistical analyses were performed using Kruskal-Wallis test. P less than 0.05 is accepted as statistically significant.

## ***Results***

### *TAK-603 reduced renal lesions in WKY rats.*

Immunofluorescence analysis revealed no deposition of rabbit IgG in glomeruli from normal WKY rats. Rabbit IgG was detected in an intense linear pattern along the glomerular capillaries from nephrotoxic serum injected rats administered TAK-603 or vehicle (data not shown).

Glomerular lesions exhibited endocapillary proliferation, severe necrotizing lesions and crescentic formation. In contrast, administration of TAK-603 drastically reduced severe crescentic lesions and necrotizing lesions, which were observed both on day 6 and 56 in control animals (Figure 2). TAK-603 prevented the development of crescents per 100 glomeruli in section (Figure 3a). The total number of glomerular cells was also reduced (Figure 3b).

On day 56, interstitial fibrosis was clearly suppressed in Group 1 and 3. Glomerular sclerosis revealed the tendency of improvement in Group 1 (Figure 4).

### *Reduced population of leukocyte populations in WKY rats.*

Numbers of both CD-4- and ED-1-positive cells were dramatically reduced with the administration of TAK-603 in all of three groups, compared with untreated rats on day 6 (Figure 3c and d).

### *Effect of TAK-603 on urinary protein secretion and electron microscopic study.*

Normal rats excreted minute amounts of protein in the urine on day 6. Proteinuria was dramatically decreased by the administration of TAK-603 in Group 1 and 3, but not in Group 2. On day 56, proteinuria was decreased in all of three groups (Figure 5a).

By electron microscopy, an extensive fusion of the foot processes around the infiltrated leukocytes was observed in untreated rats but not in Group 1, which reflects decreased proteinuria (Figure 5b and 5c).

### *Effect of TAK-603 on serum creatinine.*

Serum creatinine concentrations at day 6 were not different whether TAK-603 was administered or not. On day 56, compared with untreated rats, serum creatinine concentrations were depressed in all TAK-603 treated groups (Figure 6).

*RT-PCR analysis of cytokine in mRNA levels.*

In untreated rats,  $\gamma$ -IFN and IL-2 mRNA were elevated. After administration of TAK-603,  $\gamma$ -IFN and IL-2 mRNA were significantly downregulated in Group 1, and appeared decreasing tendency in Group 2 and 3 (Figure 7).

*Effects of TAK-603 on Urinary  $\gamma$ -IFN and IL-4.*

Urinary  $\gamma$ -IFN level was normal in normal rats. On day 6, it was elevated to 0.7pg/mlCr in untreated rats. In contrast, it was decreased to normal level by administration of TAK-603. Urinary IL-4 levels were normal in any groups (data not shown).



## ***Discussion***

In this report, we established a hypothesis that suppression of Th1 cytokines ameliorates crescentic glomerulonephritis. Therefore we investigate the therapeutic effect of TAK-603 on crescentic glomerulonephritis through the selective inhibitory effect to Th1 T cells.

Production of IgG2a and Th1 cytokines, such as IFN- $\gamma$ , IL-2, IL-12 gives a notion that glomerulonephritis is prone to Th1 dominant(15,16). The role of IFN- $\gamma$  is mostly investigated. Kitching et al. suggested that in crescentic glomerulonephritis model with C57BL/6 mouse, which is thought to Th1 dominant, genetical deficiency in IFN- $\gamma$  demonstrates suppression of crescentic formation and improvement of glomerular injury(17). Recently, IFN- $\gamma$  producing cell was found in Bone marrow transplanted IFN- $\gamma$ -deficient chimeric mice(18). These results suggests that IFN- $\gamma$  expression by both bone marrow-derived cells and intrinsic renal cells is required for the development of crescentic glomerulonephritis. For the present, there is no idea to control Th1 cells or Th1 cytokines directly. In past studies, trials that suppress crescentic glomerulonephritis by control Th1/Th2 balance were made. Tipping and Kitching et al. reported administration of Th2cytokines IL-4 and IL-10 improved glomerulonephritis(19,20). It is interesting that even after glomerulonephritis was induced, controlled Th1/Th2 balance suppressed nephritis. Indeed in our study, administration of TAK-603 from day 3 had an effect on suppress crescent formation.

Number of infiltrating CD-4- and ED-1-positive cells were clearly reduced by administration of TAK-603. The role of T cells and macrophages to crescent formation in WKY rat is reported. Wada et al. reported that administration of neutralizing antibody to monocyte chemoattractant protein-1(MCP-1)/CCL2, which acts on infiltration and activation of T cells and macrophages, improved crescent formation and other glomerular lesions, and reduced proteinuria(3). In the other reports, , MCP-1 and Th1 chemokines, such as macrophage inflammatory protein-1  $\alpha$  (MIP-1  $\alpha$ )/CCL3 and regulated on activation normal T-cell expressed and secreted(RANTES)/CCL5 may participate in activation of human crescentic glomerulonephritis (21,22). The receptor of these chemokines CCR5 specifically manifests on Th1 cells(23). And, IFN- $\gamma$  acts on mesangium cells and products MCP-1, MIP-1  $\alpha$  and RANTES(24,25). These findings give speculation that in Th1 predominance, production of chemokine from glomerular intrinsic cell and Th1 T cells induce filtration and of T cell and macrophage into

glomerulus and their activation.

In our study administration of TAK-603 also reduce amount of proteinuria. Electron microscopy findings showed that extensive fusion of the foot processes was suppressed around the infiltrated leukocytes. From this result it seems that administration of TAK-603 suppressed not only filtration of T cells and macrophage but also activation of infiltrated cells. In fact, the amount of proteinuria was mostly suppressed in Group1. In past reports, the possibility is shown that secretion of lysosome and production of superoxide from infiltrated cells induce destruction of basement membrane and directly obstruct epithelial cell and endothelial cells, and result in production of proteinuria(26,27). In our study, it seems the possibility that administration of TAK-603 suppressed activation of infiltration cells and epithelial cells injury, result in reduction of proteinuria.

WKY rats are known to be more susceptible to anti GBM antibody than other strains, and develop progressive proteinuria and crescentic glomerulonephritis(3,8). Holdsworth et al. reported that medication of neutralizing antibody to IFN- $\gamma$  or recombinant IL-4 and IL-10 reduce crescentic glomerulonephritis(15). These results suggest the possibility that administration of Th1/Th2 cytokines or these receptors leads to the treatment of crescentic glomerulonephritis.

In this study we investigated the difference of effect of TAK-603 caused by the period of administration. It is remarkable that in rats administrated TAK-603 from day3, the amount of proteinuria decreased 40%. Clinically, it is difficult to diagnose crescentic glomerulonephritis just as the disease break out. It is rarely reported that anti-inflammatory drug was administrated after glomerulonephritis broke out(28). Thus our finding seems to be valuable because it presents the possibility that suppression of Th1 cytokines may be clinically useful for crescentic glomerulonephritis. Furthermore, crescentic formation was reduced in rats administrated TAK-603 only one day when nephritis was occurred. This result suggests the importance of immediate treatment of glomerulonephritis, and Th1 dominant state is closely related with formation of early phase of glomerulonephritis, especially crescentic formation.

In our study, administration of TAK-603 avoid exacerbation of glomerulonephritis to renal failure and pathologically suppressed glomerulosclerosis. It is worth noticing this result suggests that Th1 suppression in the early phase of glomerulonephritis protects renal function in the long run. One of the mechanisms of this drug is thought to suppress

crescent formation itself. There were also reported in this rat glomerulonephritis model crescent formation correlated with renal function(3,29). Furthermore, in human glomerulonephritis, crescent formation itself regulates prognosis of renal function(30). Moreover, it seems important that administration of TAK-603 effects on reduction of proteinuria. Recently relation between renal failure and continuous proteinuria itself or ingredients in it such as albumin, iron, transferrin, lipoprotein, complement has been suggested. That is, proteinuria which includes these ingredients causes glomerular or interstitial injury directly or through physiologic active substances such as endothelin-1, transforming growth factor(TGF)- $\beta$ , activated oxygen and lysosome(31,32). From the viewpoint of the fact that proteinuria itself commonly develops renal diseases, suppression of proteinuria by TAK-603 may protect renal function in the long term.

Furthermore, in our study inhibitory effect of glomerulosclerosis in the long run was suggested. Suppression of Th1 in early phase seems to be important as regards to think of treatment of crescentic glomerulonephritis.

In our study urinary IFN- $\gamma$  level was elevated in untreated rats, and the elevation was suppressed by administration of TAK-603. This result gives the suggestion IFN- $\gamma$  plays a key role of progression of crescent formation. It is important that the wholly control of Th1 cytokines by TAK-603, which selectively inhibits Th1 T cells is beneficial to treat crescentic glomerulonephritis.

On the other side, in this study urinary IL-4 levels were not detected neither untreated groups nor treated groups. TAK-603 suppressed the production of Th1 cytokines and not that of Th2 cytokines in adjuvant arthritis rats(5). Glomerulosclerosis is mainly induced by accumulation of various extracellular matrix to mesangium lesion. It is suggested that Th2 predominance promote glomerulosclerosis and fibrosis(33,34). Thus in our study, it was important whether early suppression of Th1 with TAK-603 cause Th2 predominance, and consequently progress glomerulosclerosis and fibrosis or not. As a result, TAK-603 didn't induce Th2 predominance from the viewpoint of urinary IL-4 level on day 6. Thus it seems that this drug mainly control Th1 cytokines, and production of Th2 cytokines play little role.

In summary, we reported that suppression of Th1 cytokines in early phase may improve crescentic glomerulonephritis and protect renal function in long term. It seems important to work out strategy that controls Th1/Th2 cytokines in moderate phase of glomerulonephritis.