Apoptosis of glomerular mesangial cells induced by sublytic C5b-9 complexes in rats with Thy-1 nephritis is dependent on Gadd45γ upregulation

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The complement C5b-9 complexes can result in cell apoptosis, but the mechanism of sublytic C5b-9-mediated glomerular mesangial cell (GMC) apoptosis in Thy-1 nephritis (Thy-1N) remains largely unclear. The Gadd45 gene is involved in the cellular response to DNA damage and can promote cell apoptosis. In this study, both Gadd45γ expression patterns and pathologic changes of renal tissue were examined in rat Thy-1N. Both Gadd45γ expression and GMC apoptosis were significantly decreased in Thy-1N rats upon the depletion of complement with cobra venom factor. Our in vitro studies showed that Gadd45γ over-expression increased sublytic C5b-9-induced GMC apoptosis, while Gadd45γ gene knockdown by siRNA greatly reduced GMC apoptosis. Moreover, Gadd45γ gene silencing in vivo markedly inhibited the pathologic changes in the renal tissue of Thy-1N rats. These data suggest that Gadd45γ gene expression is involved in regulating GMC apoptosis mediated by sublytic C5b-9 in Thy-1N.

Key words: Apoptosis · Gadd45γ · Glomerular mesangial cells · Sublytic C5b-9 · Thy-1 nephritis

Supporting Information available online

Introduction

Mesangioloproliferative glomerulonephritis (MsPGN) is a disease with high incidence in humans. Proliferation of glomerular mesangial cells (GMC) in MsPGN appears to be crucial for a subsequent increase in mesangial matrix and development of glomerulosclerosis [1, 2]. Rat Thy-1 nephritis (Thy-1N), namely anti-thymocyte serum (ATS) induced nephritis, is a widely used animal model for studying human MsPGN [3, 4]. ATS administered in rats binds to Thy-1 antigen on the membrane of GMC and then activates the complement system resulting in immunopathologic injury [5–8]. Complement activation results in generation of chemotactic factors, such as C5a, and formation of the C5b-9 complex [6, 9]. Because GMC damage in Thy-1N is complement-dependent and neutrophil-independent, complement is generally regarded as the principal mediator of GMC lesions in the rats with Thy-1N. Assembly of C5b-9 can result in formation of transmembrane channels or rearrangement of membrane lipid with loss of membrane integrity. However, injury to nucleated cells by C5b-9 is almost non-lytic (sublytic) because the cell surface has many homologous restriction factors such as CD59 and MCP. Sublytic C5b-9 complexes can trigger...
diverse events including cell apoptosis, proliferation, secretion of ECM, release of cytokines and oxidants [4, 6, 9, 10]. Complement deposits are observed in the glomeruli of patients with MsPGN [1, 2]. Our previous studies have also revealed the deposition of sublytic C5b-9 complexes on GMC surfaces in Thy-1N rats [9], but the role of sublytic C5b-9 complexes in Thy-1N has not been fully elucidated.

During the progression of Thy-1N, GMC undergo two phases of change: early apoptosis/necrosis and secondary proliferation [4, 9, 11]. The mechanisms governing GMC apoptosis during the early stage of Thy-1N, including the genes involved in GMC apoptosis, remain unclear [12, 13]. The growth-arrest- and DNA-damage-inducible protein 45 (Gadd45) gene family, which encodes proteins that play important roles in controlling cell growth, has been implicated in the cellular response to DNA damage [14]. The Gadd45 family encodes three related proteins, namely, Gadd45α, Gadd45β and Gadd45γ [15]. Several studies show that Gadd45 proteins not only induce cell G2/M arrest and inhibit cell entry into S phase, but also promote cell apoptosis [16–19]. Our previous microarray analysis detected the up-regulated expression of Gadd45γ gene in the renal tissue of Thy-1N rats [20] and in GMC stimulated by sublytic C5b-9 complexes (unpublished data). However, the role of Gadd45γ gene in GMC apoptosis of Thy-1N remains to be defined.

Given that the injury of GMC in Thy-1N rats is complement-dependent and neutrophil-independent [21–25], and that the Gadd45γ gene is up-regulated during the development of Thy-1N, we aimed to examine whether the GMC apoptosis in Thy-1N is mediated by sublytic C5b-9 complexes and whether up-regulated Gadd45γ gene expression is involved in GMC apoptosis. In addition, we sought to determine whether inhibition of Gadd45γ expression can alleviate the pathologic changes such as GMC apoptosis in rats with Thy-1N.

**Results**

**Effect of cobra venom factor on complement depletion in experimental rats**

To confirm whether cobra venom factor (CVF) could deplete complement components in vivo, the activity of serum complement CH50 and deposition of glomerular C3, C5 and C5b-9 in the three groups of experimental animals were examined at different time intervals. The results showed that, although CH50 activity in the Thy-1N model group was obviously decreased owing to consumption of the nephritis, the CH50 activity in the CVF + Thy-1N group was significantly lower than that in the Thy-1N and normal human serum (NS) groups at −1 h, 40 min, 24 h and day 5 (Fig. 1A). Pronounced deposition of glomerular C3, C5 and C5b-9 was observed in the Thy-1N model group at 40 min, 3 h, 12 h and 24 h, while only mild or no deposition appeared in the CVF + Thy-1N group. No positive staining for rabbit IgG, rat C3, C5 and C5b-9 was observed in the NS group (Fig. 1B). The number of C5b-9+ cells in the CVF + Thy-1N group was markedly

![Figure 1](image-url)
lower than that in Thy-1N model group at 40 min, 3 h, 12 h and 24 h (Fig. 1C).

Effect of complement depletion on glomerular damage in Thy-1N rats

In order to assess the role of CVF in inhibiting glomerular injury of Thy-1N rat, the pathological changes of renal tissue in the three groups were examined by light microscopy and transmission EM. Glomerular cell numbers began to decrease after 40 min, and were further reduced by 24 h in the Thy-1N model group. Focal mesangial hypercellularity became conspicuous in the Thy-1N model group by day 5. The total number of glomerular cells in the CVF-1 Thy-1N group was markedly higher than in Thy-1N model group at 24 h, but substantially decreased by day 5 (Fig. 2A). Moreover, GMC in the Thy-1N rats exhibited irregular aggregation of chromatin in the periphery of the nucleus and apparent condensation of nuclear chromatin at both 3 and 6 h under EM (Fig. 2B), which demonstrated GMC apoptosis. At 24 h, some GMC began to lyse. On day 5, the glomeruli were enlarged showing mesangial hypercellularity and ECM accumulation. However, neither marked GMC apoptosis nor obvious GMC proliferation was seen in the CVF-1 Thy-1N group when compared with the Thy-1N model group at the time mentioned above. The rats in the NS group exhibited normal glomerular histology (Fig. 2B).

Effects of both complement depletion and sublytic C5b-9 attack on GMC apoptosis

Thy-1N pathology is initially associated with GMC apoptosis and necrosis, followed by secondary GMC proliferation. In the present study, we sought to examine whether complement, especially sublytic C5b-9, could lead to GMC apoptosis in Thy-1N rats. The effects of complement depletion in vivo and sublytic C5b-9 stimulation in vitro on GMC apoptosis were determined. In the Thy-1N model group, a portion of glomerular cells began to undergo apoptosis at 40 min. The apoptosing population became more prominent at 3 h. The number of TUNEL-positive cells in the glomeruli decreased gradually at 6, 12 and 24 h. The number of TUNEL-positive cells in the CVF-1 Thy-1N group was obviously reduced at these time intervals. Few TUNEL-positive cells were detected in the glomeruli of the NS group (Fig. 3A). GMC response to sublytic C5b-9 attack was assayed in culture. Flow cytometric analysis of apoptosis revealed that GMC apoptosis became detectable at 40 min and markedly increased at 3 h. Subsequently, GMC were cultured with following treatments: sublytic C5b-9, anti-Thy 1 Ab, anti-Thy1 Ab+heat-inactivated serum (HIS), anti-Thy1 Ab+human complement C6-deficient serum (C6DS) and Modified Eagle's cell culture medium (MEM). Flow cytometric analysis showed that the number of apoptotic GMC induced by sublytic C5b-9 was significantly increased compared with control groups (Fig. 3B).

Effects of complement depletion and sublytic C5b-9 attack on Gadd45γ gene expression in GMC

Our previous data revealed that expression of Gadd45γ was up-regulated in the apoptotic phase of Thy-1N [20]. Next, we examined whether the expression of glomerular Gadd45γ could be modulated by depleting complement components with CVF or, alternatively, by sublytic C5b-9 stimulation. It was found that the level of Gadd45γ mRNA in renal tissues of Thy-1N rats was up-regulated when compared with that of the NS group. Gadd45γ transcripts began to increase at 40 min, reached a peak at 3 h,
then gradually decreased from 6 h onwards (Supporting Information Fig. 7). Gadd45γ mRNA transcript levels in the CVF+Thy-1N group was markedly lower than in the Thy-1N model group, but higher than that in the NS group (Fig. 4A). Moreover, Western blot analysis confirmed that Gadd45γ protein expression in the Thy-1N model group was also up-regulated compared with the NS group. Gadd45γ protein expression did not show any change at 40 min, but was significantly increased at 3 h. Semi-quantitative analysis revealed that the level of Gadd45γ protein in the CVF+Thy-1N group at 3 h decreased when with the Thy-1N model group (Fig. 4B). Immunofluorescence analysis revealed that the glomerular expression of Gadd45γ protein at 3 h was much higher than that at 40 min and 24 h in the Thy-1N model group. However, Gadd45γ expression in the CVF+Thy-1N group was markedly inhibited at these time points. The NS group showed no positive staining for Gadd45γ protein (Fig. 4C).

Gadd45γ expression in cultured GMC after sublytic C5b-9 stimulation was determined at both mRNA and protein levels. It was found that Gadd45γ mRNA expression in the GCM induced by sublytic C5b-9 attack was up-regulated at 40 min, significantly increased at 3 h and then gradually decreased at 6, 12 and 24 h (data not shown). Gadd45γ protein expression in the GCM with sublytic C5b-9 attack emerged at 40 min, became markedly increased at 3 and 6 h, and then gradually decreased at 12 and 24 h (data not shown). Among different treatment groups assayed at 3 h, the Gadd45γ expression in the sublytic C5b-9 group was significantly increased compared with MEM, anti-Thy1 Ab+C6DS, anti-Thy1 Ab+HIS or anti-Thy1 Ab groups (Fig. 4D and E).

Figure 3. Analysis of cell apoptosis in response to complement depletion and sublytic C5b-9 attack in Thy-1N rat renal tissue and in cultured GMC.

(A) Detection of apoptotic cells by TUNEL assay (i, scale bar = 10 μm) and comparison of glomerular apoptotic cell numbers between the three groups at 3 h (ii, n = 8 every time point in each group). Data are expressed as mean ± SD. The number of apoptotic cells in the CVF+Thy-1N group was markedly less than that in the Thy-1N model group and higher than that in the NS group at all time points assayed (**p<0.01 versus the Thy-1N model group, Δp<0.01 versus the NS group). (B) Numbers of apoptotic GMC in the sublytic C5b-9, anti-Thy1 Ab, anti-Thy1 Ab+HIS, anti-Thy1 Ab+C6DS and MEM groups at 3 h, analyzed by flow cytometry (i, n = 4 in each group). The number of apoptotic GMC in sublytic C5b-9 group significantly increased (ii, **p<0.01 versus MEM, Δp<0.01 versus anti-Thy1 Ab+C6DS, ΔΔp<0.01 versus anti-Thy1 Ab+HIS, *p<0.01 versus anti-Thy1 Ab). Data show mean ± SD. Comparisons between different doses were analyzed by ANOVA and post hoc Bonferroni correction.

Role of Gadd45γ gene over-expression in GMC apoptosis induced by sublytic C5b-9

To further determine the effect of Gadd45γ over-expression on the sublytic C5b-9-induced cell apoptosis, either Gadd45γ-expressing plasmids (pGadd45γ) or an expression vector control (pcDNA3.1) were transfected into GMC. GMC were subsequently stimulated by sublytic C5b-9 complexes. The cultured GMC were divided into six groups: pGadd45γ+sublytic C5b-9 group, pcDNA3.1+sublytic C5b-9 group, sublytic C5b-9 group, pGadd45γ group, pcDNA3.1 group and culture medium (MEM) group. The results showed that the expression of Gadd45γ in the pGadd45γ group was markedly up-regulated, compared with the MEM or pcDNA3.1 groups. The level of Gadd45γ expression in the pGadd45γ+sublytic C5b-9 group was higher than that of the pGadd45γ, sublytic C5b-9 or pcDNA3.1+sublytic C5b-9 groups by real-time PCR and Western blot analyses (Fig. 5A and B). In addition, over-expression of Gadd45γ was observed to induce GMC apoptosis. The number of apoptotic GMC in pGadd45γ+sublytic C5b-9 group was also significantly higher than that in pGadd45γ, sublytic C5b-9 and pcDNA3.1+sublytic C5b-9 groups, although the pGadd45γ, sublytic C5b-9 and pcDNA3.1+sublytic C5b-9 group displayed increased GMC apoptosis (Fig. 5C).
Figure 4. Detection of Gadd45γ expression after complement depletion and sublytic C5b-9 attack in renal tissue and cultured GMC. (A) Gadd45γ mRNA expression in the CVF+N Thy-1N group were markedly lower than in the Thy-1N model group at 40 min and 3 h (**p<0.01), but greater than that observed in the NS group (*p<0.05). Data show mean ± SD (n = 8). (B) Gadd45γ protein expression in the Thy-1N model, CVF+N Thy-1N and NS groups at 40 min and 3 h. Semi-quantitative analysis showed that the Gadd45γ protein levels in CVF+N Thy-1N group at 3 h were lower than that in Thy-1N model group (**p<0.01), but higher than that in NS group (*p<0.05). Data show mean ± SD (n = 4). (C) Immunofluorescence analysis of Gadd45γ protein expression in glomeruli. Photomicrographs are representative of glomeruli from the three groups at each time intervals. Gadd45γ protein expression appeared at 40 min, peaked at 3 h and then decreased in the Thy-1 model group, whereas only faint Gadd45γ expression was detected in the CVF+N Thy-1N group. No fluorescence was detected in the NS group at the indicated time (scale bar = 10 μm). (D) Levels of Gadd45γ mRNA in sublytic C5b-9 group at 3 h were significantly higher compared with other groups by real-time PCR analysis (**p<0.01 versus MEM, ##p<0.01 versus anti-Thy1 Ab+C6DS, &&p<0.01 versus anti-Thy1 Ab+HIS, *p<0.01 versus anti-Thy1 Ab). Data show mean ± SD. (E) Photomicrographs showing Gadd45γ protein expression at 3 h in sublytic C5b-9, anti-Thy1 Ab, anti-Thy1 Ab+HIS, anti-Thy1 Ab+C6DS and MEM groups. Semi-quantitative analysis showed that Gadd45γ protein in sublytic C5b-9 group was markedly up-regulated, compared with other groups (**p<0.01 versus MEM, ##p<0.01 versus anti-Thy1 Ab+C6DS, &&p<0.01 versus anti-Thy1 Ab+HIS, *p<0.01 versus anti-Thy1 Ab). Data show mean ± SD. Comparisons between different doses were analyzed by ANOVA and post hoc Bonferroni correction.
Effect of Gadd45α gene knockdown on GMC apoptosis induced by sublytic C5b-9

GMC cultured under various treatments were transfected with Gadd45α small hairpin RNA (shRNA) for 48 h to knock down the expression of Gadd45α gene. After sublytic C5b-9 attack for 3 h, both mRNA and protein levels of Gadd45α in the four groups were determined by real-time PCR or Western blot analyses. GMC apoptosis was assayed by flow cytometry. The levels of Gadd45α mRNA and protein in the GMC transfected with Gadd45α shRNA followed by sublytic C5b-9 stimulation were significantly decreased in comparison with control shRNA + sublytic C5b-9 or sublytic C5b-9 only groups (Fig. 6A and B). Moreover, similar changes in GMC apoptosis among the four groups were observed (Fig. 6C), suggesting that the Gadd45α gene knockdown in the GMC can markedly suppress GMC apoptosis induced by sublytic C5b-9 attack.
Effect of Gadd45γ gene knockdown on GMC pathologic changes in Thy-1N rats

In order to further confirm the role of Gadd45γ in regulating GMC apoptosis in rats with Thy-1N, normal Sprague–Dawley (SD) rats were divided into six groups with different treatments, namely, (i) Gadd45γ shRNA+Thy-1N group, (ii) control shRNA+Thy-1N group, (iii) pGadd45γ group, (iv) pcDNA3.1 group, (v) Thy-1N model group and (vi) NS group. It was found that the level of Gadd45γ protein in the Gadd45γ shRNA+Thy-1N group at 3 h was markedly down-regulated compared with the control groups (shRNA+Thy-1N and Thy-1N model groups) (Fig. 7A). These results were verified by immunofluorescence staining for Gadd45γ protein in the glomeruli (Fig. 7B). On the other hand, the number of TUNEL-positive cells was substantially reduced at 3 h in Gadd45γ shRNA+Thy-1N group, when compared with control shRNA+Thy-1N and Thy-1N model groups, and few TUNEL-positive cells were detected in the glomeruli of the NS group.
Over-expression of Gadd45γ gene in the rat kidney (pGadd45γ group) induced GMC apoptosis, but transfection of pcDNA3.1 into renal tissue did not (Fig. 8A). EM revealed reduced pathological changes, such as irregular aggregation of chromatin in the periphery of the nucleus and clear condensation of the nuclear chromatin, in the Gadd45γ shRNA1 Thy-1N group at 3 h when compared with controls. Normal glomerular ultrastructures were observed in the NS group rats. Rat renal tissue transfected with pGadd45γ showed irregular aggregation of chromatin and condensation of the nuclear chromatin. Transfection of pcDNA3.1 into kidney tissue (pcDNA3.1 group) did not induce the proliferation of glomerular cells (Fig. 9A and B).

Effects of Gadd45γ gene knockdown on the excretion of urinary protein in Thy-1N rats

The content of urinary protein excretion (mg/24 h) in the Gadd45γ shRNA1 Thy-1N group was lower than that in control shRNA+Thy-1N (p<0.01) and Thy-1N model groups (p<0.01). Meanwhile, Gadd45γ protein expression in the pGadd45γ group was more than that in pcDNA3.1 (p<0.01) and NS groups (p<0.01). Data show mean ± SD (n = 8). (B) Immunofluorescence analysis of Gadd45γ protein expression in glomeruli from the six treatment groups at 3 h. Only a faint positive reaction for Gadd45γ protein was seen for the Gadd45γ shRNA+Thy-1N group, but in control shRNA+Thy-1N, pGadd45γ and Thy-1N model groups, the presence of Gadd45γ protein was marked. No fluorescence was seen in pcDNA3.1 group and NS group at the same time (scale bar = 10 μm). Photos are representative of glomeruli in each group. Comparisons between different doses were analyzed by ANOVA and post hoc Bonferroni correction.

Discussion

Rat Thy-1N, an animal model of human immune-mediated MsPGN, is characterized by a series of immunologic events [3, 4]. In the early phase of Thy-1N, GMC are targeted by anti-Thy-1 Ab resulting in immediate complement activation and cell injury [6]. There is growing interest in studying GMC apoptosis and its involvement in the initiation and development of the nephritis [11, 20, 21]. Shimizu et al. [4] reported that GMC apoptosis in the rats with Thy-1N occurs rapidly (within 1 or 2 h). In the present study, an increased number of TUNEL-positive cells was found 40 min after i.p. injection of ATS, peaking at 3 h and then gradually decreasing. Damaged GMC showed typical morphology of apoptosis, including condensation and shrinkage of nuclei, at 3 and 6 h. There was a decrease in total glomerular cellularity and a marked reduction in the number of TUNEL-positive GMC at 24 h. With the progression of Thy-1N, secondary GMC proliferation and ECM accumulation were seen by day 5. Our results provide strong evidence that Thy-1N...
disease development initially involves GMC apoptosis and necrosis, and finally, secondary cell proliferation.

Complement activation is a pathogenic feature in human and animal renal diseases [26–31]. Occurrence of the complement cascade near the cell surface can lead to assembly of terminal components (C5b-9) [28, 29, 32–36]. In Thy-1N, the anti-Thy-1 Ab binds to the corresponding antigen located on the surfaces of GMC and forms immune complex, then activates the complement system leading to C5b-9 formation in the cell membrane [37, 38]. Early studies of Thy-1N have demonstrated that the damage of GMC is complement-dependent and neutrophil-independent [6, 9]. Complement C5b-9, in particular sublytic C5b-9, can induce sublethal injury and activate various metabolic processes including expression of many pathogenic genes [9, 20, 22, 23]. Subsequent experiments have confirmed that systemic complement depletion can reduce mesangiolysis, GMC proliferation and glomerular platelet and macrophage influx [21]. Brandt et al. [6] have reported that the total absence of C6 in C/C0 PVG rats (complement C6-deficient rat) induces no glomerular injury after injection of anti-Thy-1 Ab because C/C0 PVG rats are unable to generate detectable C5b-9 following complement activation. Attack of sublytic C5b-9 to GMC may be crucial to the induction of GMC apoptosis or proliferation observed during Thy-1N [10, 23]. However, the role and mechanism of sublytic complement C5b-9 on GMC apoptosis, including the expression of apoptosis-related genes, has not been well elucidated.

Figure 8. Renal pathological changes during disease progression under conditions of Gadd45g gene knockdown (n = 8 in each group). (Ai) Photomicrographs of glomerular TUNEL-positive cells at 3h (scale bar = 10 μm). (Aii) The number of TUNEL-positive cells in the Gadd45g shRNA+Thy-1N group was markedly lower than that in the control shRNA+Thy-1N (p < 0.01) and Thy-1N model groups (p < 0.01). In addition, the number of TUNEL-positive cells in the pGadd45g group was more than that in pcDNA3.1 (p < 0.01) and NS groups (p < 0.01). Photomicrographs are representative of rat glomeruli in each of the six groups at 3h. Data show mean ± SD. (B). Ultrastructural changes detected by EM in GMC (scale bar = 2.5 μm). The photomicrographs are representative of rat glomeruli in each of the six groups at 3h. The pathological changes in Gadd45g shRNA+Thy-1N group, including irregular aggregation of chromatin at the periphery of the nucleus and condensation of the nuclear chromatin (arrow) were reduced compared with control shRNA+Thy-1N and Thy-1N model groups. In pGadd45g group, apoptotic morphology including condensation of the nuclear chromatin (arrow) was obvious compared with pcDNA3.1 or NS group. Comparisons between different doses were analyzed by ANOVA and post hoc Bonferroni correction.
Gadd45 is a family of genes that are implicated in the cellular response to DNA damage [39–41]. Gadd45α, β and γ (also termed Gadd45, MyD118 and CR6) comprise the family of Gadd45 genes that encode for proteins that play important roles in negative growth control [16]. Accumulated data indicate that Gadd45α and γ serve similar functions along different apoptotic and growth suppressive pathways [17, 18]. All three Gadd45 isoforms not only induce cell G2/M arrest, inhibit entry of cells into S phase and cell proliferation, but also stimulate DNA repair and promote apoptosis in certain cells exposed to environmental stress [42]. Gadd45 binds to proliferating cell nuclear antigen at several residues, thereby causing cell cycle arrest. Although Gadd45α/b induction can induce cell apoptosis under some conditions, the Gadd45γ gene acts as a critical and essential promoter of cell apoptosis at all times [16, 18, 43]. Studies by Pippin et al. demonstrated that DNA damage was a novel response to sublytic complement C5b-9-induced injury in podocytes [42]. Our previous studies using gene microarray analysis revealed that the expression of Gadd45γ gene was up-regulated in the renal tissue of Thy-1N rats and in GMC stimulated by sublytic C5b-9 (unpublished data). Both mRNA and protein expression of Gadd45γ detected by real-time PCR and IHC staining in the early phase of the GMC apoptosis in Thy-1N are remarkably increased [20], suggesting that up-regulation of Gadd45γ expression may play a role in the GMC apoptosis. It is possible that the up-regulation of Gadd45γ expression may play a role in the GMC apoptosis. On the other hand, over-expression of Gadd45γ seems to be a very early marker of the GMC apoptosis in Thy-1N. Given that GMC damages in Thy-1N are dependent on sublytic C5b-9 complexes [4, 6, 9], and that Gadd45γ gene expression is simultaneously up-regulated in vivo and in vitro as revealed by our previous studies [44], the relationship between complement C5b-9 and Gadd45γ, including the role of Gadd45γ in the GMC apoptosis induced by sublytic C5b-9 complexes, are worthy of further investigation.

In Thy-1N, the initial complement-dependent GMC injury is followed by a phase of marked mesangial proliferation.

![Figure 9](image1.png) **Figure 9.** Morphology changes in glomerular cells after Gadd45γ gene knockdown. (A) Photomicrographs of H&E-stained glomeruli day 5 after knockdown (scale bar = 10 μm). (B) Quantification of glomerular cell density under the six treatment modalities (n = 8 in each group). The number of the glomerular cells in the Gadd45γ shRNA+Thy-1N group was obviously less than that in the control shRNA+Thy-1N (p<0.01) and Thy-1N model group (p<0.01). The number of glomerular cells in the pGadd45γ group was higher than that in pcDNA3.1 (p<0.01) and NS groups (p<0.01). Data show mean±SD. Comparisons between different doses were analyzed by ANOVA and post hoc Bonferroni correction.

![Figure 10](image2.png) **Figure 10.** Effect of Gadd45γ gene knockdown on the excretion of urinary protein in Thy-1N rats. The content of urinary protein excretion (mg/24 h) in the Gadd45γ shRNA+Thy-1N group was lower than that in the control shRNA+Thy-1N (p<0.01, n = 8 in each group) and Thy-1N model groups (p<0.01), although it was still higher than that in the NS group (p<0.01) at 5 d. Urinary protein in the pGadd45γ group was markedly more abundant than that in pcDNA3.1 (p<0.01) and NS groups (p<0.01), and there was no significant difference of urinary protein excretion between pcDNA3.1 and NS groups (p>0.05). Data show mean±SD. Comparisons between different doses were analyzed by ANOVA and post hoc Bonferroni correction.
study, we provide evidence that the administration of CVF prior to
the initiation of Thy-1N and during Thy-1N to deplete complement
components can greatly reduce the pathological changes such as
GMC apoptosis, GMC secondary proliferation and ECM secretion.
Moreover, the expression of glomerular Gadd45γ in Thy-1N rats
treated with CVF is significantly decreased compared with Thy-1N
model group, implying that inhibition of complement activation
regulates the expression of Gadd45γ gene in the renal tissue. Our
findings support a notion that up-regulation of glomerular
Gadd45γ expression in the rats with Thy-1N is related to the
activation of complement system and that the pathologic changes,
including the early injury of glomerular cells and subsequent
proliferative response, are both complement-dependent.

Several studies have reported that the injury of the GMC in
Thy-1N is complement-dependent, especially sublytic C5b-9
complexes [4, 6, 9, 21], and in our experiment, the formation of
sublytic C5b-9 complexes on the surface of GMC was observed
during early stages of rat Thy-1N (Fig. 1B). In order to explore
the role of sublytic C5b-9 complexes in GMC apoptosis during the
development of Thy-1N, we measured Gadd45γ gene expression
and GMC apoptosis in the cultured GMC incubated in the
presence of sublytic C5b-9 complexes. Our studies in vitro
revealed that GMC apoptosis and Gadd45γ gene expression in
the GMC are increased by sublytic C5b-9 attack. Over-expression
of Gadd45γ protein enhanced GMC apoptosis mediated by sublytic
C5b-9. Knockdown of Gadd45γ gene with Gadd45γ shRNA
inhibited GMC apoptosis in response to sublytic C5b-9 attack. In
vivo, glomerular Gadd45γ gene over-expression could induce
GMC apoptosis in rat kidneys. In contrast, glomerular Gadd45γ
gene silencing using shRNA could effectively alleviate pathologic
changes of renal tissue such as GMC apoptosis, proliferation and
production of urinary protein in Thy-1N. Taken together, these
results suggest that sublytic complement C5b-9 complexes can
induce GMC apoptosis, which is partially mediated via increased
Gadd45γ gene expression.

In summary, we have demonstrated that the GMC apoptosis,
accompanied by up-regulation of Gadd45γ expression during the
early stage of Thy-1N, is complement-dependent. Furthermore,
sublytic complement C5b-9 complexes in vitro could induce GMC
apoptosis through up-regulated Gadd45γ expression. Overall,
these findings indicate that GMC apoptosis induced by sublytic
C5b-9 complexes in Thy-1N is partially dependent on up-regula-
tion of Gadd45γ expression.

Materials and methods

Reagents and animals

Polyclonal Ab against Gadd45γ were purchased from Santa Cruz
Biotechnology (Santa Cruz, USA). CVF was supplied by Kunming
Institute of Zoology (Kunming, China). Monoclonal Ab against
rat C3, C5 and C5b-9 were purchased from Santa Cruz
Biotechnology. Polyclonal Ab against rabbit IgG were purchased
from Southern Biotech (Birmingham, USA). Monoclonal Ab
against human influenza hemagglutinin (HA) was supplied by
TIANGEN (Beijing, China). HRP or Cy5-linked anti-goat secondary
Ab were from Jackson ImmunoResearch Laboratories (West
Grove, USA). For Western blot analysis, HRP-conjugated anti-
mouse IgG Ab, 20 × LumiGLO reagent and 20 × peroxide were
purchased from Cell Signaling Technology (Danvers, USA). The
TUNEL staining kit was obtained from Roche (Basel, Switzer-
land). TRIZOL reagent was purchased from Invitrogen (Carlsbad,
USA). Moloney murine leukemia virus was provided by Promega
(Madison, USA). shRNA and pGCsi were obtained from Shanghai
Genkan Biotechnology (Shanghai, China). The pcDNA3.1 vector
with a HA tag was a gift from Dr. Jianmin Li in Nanjing Medical
University (Supporting Information Fig. 1). The restriction
enzyme Sfi I and T4 DNA ligase were purchased from TaKaRa
(Tokyo, Japan). QIAprep spin miniprep kit was obtained from
QIAGEN (Hilden, Germany). For apoptosis assays, annexin V-
APC was purchased from Bender MedSystems (Vienna, Austria).

SD rats were from Laboratory Animal Center of Nanjing
Medical University, and New Zealand white (NZW) male rabbits
were provided by BK (Shanghai, China). An institutional approval
for the animal study protocol was obtained. Rat GMC strain
was provided by China Centre for Type Culture Collection (Wuhan,
China). MEM and FBS were obtained from Gibco (Carlsbad, USA).
NS from several healthy adult donors were used as a source of
serum complement, and HIS was obtained by incubating the NS at
56 °C for 30 min. Human C6DS was obtained from Sigma
(St. Louis, USA). Rabbit polyclonal Ab (anti-Thy1 Ab, titer 1:640)
against Thy-1 antigen of rat thymocyte, namely the ATS, was
prepared according to previously published procedures [7, 9].

Construction of Gadd45γ expression plasmid

The expression plasmid of pcDNA3.1-Gadd45γ (pGadd45γ) was
constructed by inserting the complete ORF of rat Gadd45γ cDNA
(89 ~ 568) into the mammalian expression vector (pcDNA3.1
with a HA tag). In brief, the first strand cDNA was synthesized
from total RNA of the cultured GMC using M-MLV-RT. The
Gadd45γ gene was amplified by PCR. Specific primers were
obtained from Invtrogen and, the sequence was as follows:
forward primer, 5’-AAGGGAATTCGGCCATGACTCTGG-
AAGAAGTGCG-3’, reverse primer, 5’- GCCTCTAAAGGGCT-
CAGTCGGAAGGATGC-3’. PCR products and pcDNA3.1
division was ligated using T4 DNA ligase. PCR products were
amplified with Sfi I and ligated using T4 DNA ligase. The
recombinant plasmids were amplified in E. coli strain DH5α
and purified with QIAprep spin miniprep kit. The constructed
plasmid was sequenced across both junctions to confirm the
nucleotide sequence and the predicted orientation [45].

Generation of Gadd45γ shRNA-expressing vector

To ensure the silencing of target genes, four different siRNA
sequences were designed to be homologous to Gadd45γ

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consensus sequence. The sequence of Gadd45γ from the Rattus norvegicus growth arrest and DNA damage-inducible 45 gamma, complete cds (GenBank accession number NM_001077640) was chosen as shGadd45γ target genes. The complementary oligonucleotides encoded a hairpin structure with a 21-mer stem derived from the target site. The vectors of shGadd45γ-expressing plasmid were constructed using pGCsi (Supporting Information Fig. S2) as the vector backbone while a scrambled gene shRNA was chosen as a negative control [46].

Culture of GMC and determination of sublytic C5b-9 complexes

The rat GMC were maintained in MEM medium supplemented with 10% FBS and used from five to seven passages for experiments. We used heterologous complement (NS) to minimize possible signaling via complement-regulatory proteins as in previous studies [22, 24]. To ensure that C5b-9 attack was insufficient to induce marked cell lysis, lactate dehydrogenase (LDH) was measured in the supernatants of cultured cells using an LDH Assay Kit (Nanjing Jiancheng Bioengineering Institute, China) [35, 47]. Lysis rate was expressed as a percentage of the change in the absorbance of the media compared with changes in absorbance of the triton lysed cells and the media. Less than 5% LDH release from cells was regarded as a sublytic effect [22, 35, 47]. The selection of anti-Thy 1 Ab (ATS) and complement concentration used in this study was 5% ATS and 4% NS with less than 5% LDH release. To confirm that the effects on GMC following above stimulation were in fact due to sublytic C5b-9, control cells were treated identically with 5% ATS, 5% ATS+4% HIS, 5% ATS+4% C6DS or MEM [22, 23, 47].

GMC transfection and identification

Transient transfection of pGadd45γ or Gadd45γ shRNA expression plasmid into the cultured GMC was conducted with GenEscort™III [48, 49] according to the manufacturer’s instructions. Briefly, 4.0 μg plasmid and 20 μL transfection reagent were incubated in 0.4 mL PBS for 12 min. The resulting mixture was added to the cells cultured in 6-well plates with 0.8 mL of complete media. After incubation for 5 h, cell samples were re-fed with 1 mL complete media and left for 48 h. The cells were then homogenized with lysis buffer and the expression of HA protein was assessed by Western blot analysis (Supporting Information Fig. 3A). To further detect HA protein by immunohistochemistry, the cells were incubated with an anti-HA Ab, then with HRP-conjugated anti-mouse IgG Ab and visualized with DAB (Supporting Information Fig. 3B) [50]. The efficiency of shRNA transfection was examined by the fluorescence of GFP (Supporting Information Fig. 4). As for Gadd45γ shRNA, the four classes of shRNA to different target sites of Gadd45γ gene were designed and transfected into the GMC to repress the target gene. Gadd45γ protein expression in the GMC after transfecting shRNA was detected by Western blot (Supporting Information Fig. 5). Upon screening, the shRNA (aaAAGACTG-CACTGTCCTTCAA) that could effectively silence the Gadd45γ gene were chosen for the further study while the shRNA with scrambled sequence (AAGTACGCAGCTACCATTTCT) was used as negative control.

Rat Thy-1N model and experimental design

In order to confirm the CVF effects on the pathologic changes of rat with Thy-1N, normal male SD rats (160–200 g) were randomly divided into three groups (n = 8 every time point in each group): (i) Thy-1N model group: animals were given ATS (0.75 mL/100 g body wt.) by a single i.p. injection. (ii) CVF+Thy-1N group: the rats were injected with CVF (15 U/100 g body wt./day) i.p. in three divided doses beginning at 24 h prior to injection of ATS (0.75 mL/100 g body wt.), followed by daily injection of 10 U/rat i.p. during the experiment [21]. (3) NS group: the rats were injected i.p. with normal rabbit serum (0.75 mL/100 g body wt.). Tissue samples of the renal cortices were obtained at 40 min, 3 h, 6 h, 12 h, 24 h and on day 5 after serum injection, then embedded in paraffin and OCT as well as Epon 812.

To examine the role of Gadd45γ in the GMC apoptosis of rats with Thy-1N, normal male SD rats (160–200 g) were divided into six groups (n = 8 every time point in each group), (i) Gadd45γ shRNA+Thy-1N group, (ii) control shRNA+Thy-1N group, (iii) pGadd45γ group, (iv) pcDNA3.1 group, (v) Thy-1N model group, (vi) NS group. Rats in the Thy-1N model and NS groups were treated with the same methods described above. Rats allocated to Gadd45γ shRNA+Thy-1N, control shRNA+Thy-1N, pGadd45γ and pcDNA3.1 groups were treated with a modified method. In these rats, plasmids were transferred into kidneys via renal artery perfusion on day 3 before injection of ATS. Briefly, the left kidney and renal artery were surgically exposed with a mid-line incision. After the proximal site of the abdominal aorta was clamped, the left kidney was infused with balanced salt solution (BSS) via the renal artery. Thereafter, the plasmid solution (100 μg/100 g body wt. in 750 μL) was infused into the left kidney via scalp acupuncture (24G) and the renal vein clamped immediately after injection with bulldog clamps. The left kidney was then sandwiched between a pair of oval-shaped electrodes (WJ-2002, Ningbo Scientz Biotechnology, China), and electric pulses were delivered (six 75 V pulses of 100 ms duration each with 900 ms intervals). Following electroporation, the scalp acupuncture was removed and the bulldog clamps were released [51, 52]. The same operation was carried out in the right kidney. The cortexes of all rats were collected at 3 h and day 5 after transfection. The efficiency of transferring plasmids into the glomeruli was examined by determining the expression of GFP and HA protein (Supporting Information Fig. 6).
Evaluation of complement depletion

Rat serum samples were obtained at −1 h, 40 min, 24 h and day 5 after ATS or NS administration. The complement hemolytic activity (CH50) was determined by red cell hemolytic test [53]. Frozen renal sections prepared at 40 min, 3 h, 12 h and 24 h post serum injection were examined to detect glomerular deposits of rabbit IgG, rat C3, C5 and C5b-9 by immunoperoxidase technique as described previously [9]. For each animal, 50 glomerular cross sections were analyzed. The glomerular C5b-9 protein was visualized in three groups at fixed time intervals.

Western blot analysis for Gadd45γ protein

Rat renal tissues or cultured GMC were homogenized using Trizol reagent and purified with QIAGEN reagent kit (Hilden, Germany). The quantification of Gadd45γ gene expression was performed by real-time PCR through ABI PRISM 7300 sequence detection system with the following thermal cycling conditions: 2 min at 50°C, 10 min at 95°C followed by a total of 40 cycles of 15 s at 95°C then 1 min at 60°C. Rat β-actin was co-amplified as an internal control. Each sample was assayed in triplicate. For Gadd45γ a fluorescence-labeled probe was used (forward primer: 5'-CGTCTACAGGTCCGCCAAA-3', reverse primer: 5'-CATCG-GAACCGCTCATTTGGCCGATAG-3', FAM/TAMRA-labeled ACGCGCTCCCCATGGCATTCTGCGT-3'). β-actin was quantified using (forward prime: 5'-TCACCCACACTGTGCCCCATCTATGA-3'; reverse primer: 5'-CATCGGAAACCGCTCATTTGGCCGATAG-3'; FAM/TAMRA-labeled probe: 5'-ACGGGCCTCCCCATGCCATCTGCGT-3'). The relative level of the gene expression was obtained by calculating ratio of cycle numbers of the initial exponential amplification phase as determined by the sequence detection system for specific target gene and β-actin using the delta-delta Ct method [54].

Immunofluorescence staining for Gadd45γ protein in renal tissue

Paraffin-embedded renal tissue samples were examined to detect Gadd45γ protein. First, all sections were deparaffinized. After being washed with PBS and incubated with 5% normal rabbit serum (1:20) for 30 min to reduce non-specific background staining, the sections were incubated with purified goat anti-Gadd45γ polyclonal Ab diluted 1:200. Then the sections were incubated with the secondary Ab (Cy5-labelled rabbit anti-goat Ab). Gadd45γ protein was visualized via the blue fluorescence. For each animal, 50 glomerular cross sections were observed.

Examination of renal pathologic changes

Paraffin sections of renal tissues were stained with H&E. The glomerular cellularity was evaluated under light microscopy by counting the total number of nuclei. Numbers of glomerular cells for each rat were quantified from counts of positive-stained nuclei in hematoxylin-stain, performed in a double-blinded manner by two independent observers counting. The numbers of glomerular cells were expressed as the mean number per glomerular cross section as described previously [29, 57]. The kidney ultrathin sections were stained with uranyl acetate and lead citrate. The ultrastructural changes (especially GMC apoptosis) of the sections were examined by transmission EM [4].

TUNEL analysis of glomerular cells

Tissue sections were treated with 50µl proteinase K (0.1 mg/mL) for 10 min at 37°C. After three washes with 0.1M PBS, the sections were incubated with 50 µl reaction mixture of the TUNEL for 60 min at 37°C in the dark. After three washes using 0.1 M PBS, the samples were analyzed by fluorescence microscopy. To quantify glomerular apoptotic cells, the number of TUNEL-positive nuclei in 100 glomerular cross sections was counted in a double-blinded manner by two independent observers for each rat. Numbers of glomerular apoptotic cells were expressed as the mean number per glomerular cross section as described previously [20, 29, 57].

Apoptosis detection of cultured GMC by flow cytometry

Approximately 5 × 10^5 GMC were washed with PBS and resuspended in binding buffer containing Annexin V-APC and propidium iodide. The samples were analyzed on a FACScan flow cytometer (Becton Dickinson, San Jose, USA). The percentage of apoptotic cells in a 10,000-cell cohort was determined by flow cytometry. Each sample was assayed in triplicate.
Urine protein excretion

The rat urine samples in the six groups were collected on day 5 after different treatments. The contents of urinary protein of rats were examined using 3% sulfosalicylic acid method [9, 21]. Each sample was assayed in triplicate.

Statistical analysis

All data are given as mean ± SD. The statistical significance (defined as $p<0.05$) of the groups was evaluated by one-way ANOVA with simultaneous multiple comparisons between groups by the Bonferroni method.

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References


Abbreviations:  
ATS: anti-thymocyte serum  
C6DS: complement C6-deficient serum  
CVF: cobra venom factor  
ECM: extracellular matrix  
Gadd45γ: growth-arrest- and DNA-damage-inducible protein 45 gamma  
GMC: glomerular mesangial cell  
HA: human influenza hemagglutinin  
HIS: heat-inactivated serum  
LDH: lactate dehydrogenase  
MEM: Modified Eagle’s cell culture medium  
MsPGN: mesangioproliferative glomerulonephritis  
NS: normal human serum  
pGadd45γ: Gadd45γ-expressing plasmids  
SD rats: Sprague-Dawley rats  
sublytic C5b-9: sublytic complement C5b-9 complexes  
Thy-1N: Thy-1 nephritis

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