Activating transcription factor 3 (ATF3) promotes sublytic C5b-9-induced glomerular mesangial cells apoptosis through up-regulation of Gadd45α and KLF6 gene expression

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ABSTRACT

The sublytic C5b-9 complexes can result in glomerular mesangial cells (GMCs) apoptosis, which involved in the initiation and development of rat Thy-1 nephritis. Activating transcription factor 3 (ATF3) is an immediate early gene for cells to cope with a variety of stress signals, and our previous study revealed that ATF3 could promote GMCs apoptosis attacked by sublytic C5b-9. But the mechanism of ATF3 promoting GMCs apoptosis triggered by sublytic C5b-9 attack has not been elucidated. In this study, the data showed that the expression of ATF3, growth arrest and DNA damage-45 alpha (Gadd45α), Krüppel-like factor 6 (KLF6) and proliferating cell nuclear antigen (PCNA) in the GMCs in response to sublytic C5b-9 stimulation for the indicated time was significantly increased, and ATF3 expression could lead to GMCs apoptosis through up-regulation of Gadd45α and KLF6, but not up-regulation of PCNA. Furthermore, Gadd45α was identified as a downstream target gene regulated by ATF3 directly, and KLF6 might be regulated by ATF3 in an indirect manner.

Introduction

Mesangioproliferative glomerulonephritis (MsPGN), characterized by damage of glomerular mesangial cells (GMCs) and excessive secretion of extracellular matrix (ECM), is a disease of high incidence in humans (Berthoux et al. 2008; Yamamoto and Wilson 1987). Rats Thy-1 nephritis (Thy-1N) is a well-known model of human MsPGN (Yamamoto and Wilson 1987), and complement is generally regarded as the principal mediator of GMCs lesions in the rats with Thy-1N (Bohana-Kashlan et al. 2004; Brandt et al. 1996). It has been reported that injury to nucleated cells by complement C5b-9 is almost non-lytic (sublytic) because of homologous restriction factors on the cell surface, such as CD59 and MCP. Previous studies have revealed that sublytic C5b-9 can lead to GMCs apoptosis/necrosis and secondary proliferation in the progression of Thy-1N (Brandt et al. 1996; Cole and Morgan 2003; Gao et al. 2006; Qiu et al. 2009; Shimizu et al. 2000; Wang et al. 2006). There is growing interest in studying GMCs apoptosis and their involvement in the initiation or development of the nephritis (Amore and Coppo 2000; Qiu et al. 2009; Shimizu et al. 2000; Xu et al. 2006), but the mechanisms governing GMCs apoptosis during the early stage of Thy-1N remain unclear.

Activating transcription factor 3 (ATF3), a member of the ATF/cyclic AMP response element-binding (CREB) family, contains a basic region/leucine zipper DNA-binding motif and binds to the ATF/CRE consensus sequence TGACGTCA (Hai and Hartman 2001). Overwhelming evidence indicates that ATF3 can be induced rapidly by a variety of stress stimuli in different cell types (Ameri et al. 2007; Jiang et al. 2010; Kawauchi et al. 2002; Kool et al. 2003; Mungrue et al. 2009; Tamura et al. 2005; Turchi et al. 2009; Yin et al. 2008). Although ATF3 has dual effects on cell fate, i.e., apoptosis (Cai et al. 2000; Fan et al. 2002; Kang et al. 2003; Li et al. 2008; Lu et al. 2006; Turchi et al. 2009) or cell proliferation (Allan et al. 2001; Kawauchi et al. 2002; Nakagomi et al. 2003; Perez et al. 2001; Tamura et al. 2005), our results showed that the expression of ATF3 was significantly increased both in renal tissue of rats with Thy-1N in vivo and in cultured primary rat GMCs induced by sublytic C5b-9 in vitro, and ATF3 was involved in promoting rat GMCs apoptosis attacked by sublytic C5b-9 (Jiang et al. 2010).

Despite sublytic C5b-9 triggering and ATF3 promoting GMCs apoptosis, the mechanisms of ATF3 enhancing GMCs apoptosis have not been fully elucidated. As a transcription factor, ATF3 must exert its action, at least partly, by regulating downstream target genes, such as IL-6, C/EBP homologous protein, NOXA, CCL4, fibronectin 1, and so on (Khuu et al. 2007; Litvak et al. 2009; Qiu et al. 2009; Shimizu et al. 2000; Xu et al. 2006). Our previous study revealed that ATF3 could promote GMCs apoptosis attacked by sublytic C5b-9. But the mechanism of ATF3 promoting GMCs apoptosis triggered by sublytic C5b-9 attack has not been elucidated. In this study, the data showed that the expression of ATF3, growth arrest and DNA damage-45 alpha (Gadd45α), Krüppel-like factor 6 (KLF6) and proliferating cell nuclear antigen (PCNA) in the GMCs in response to sublytic C5b-9 stimulation for the indicated time was significantly increased.

Keywords:
MsPGN, mesangioproliferative glomerulonephritis; GMCs, glomerular mesangial cells; ECM, extracellular matrix; Thy-1N, Thy-1 nephritis; ATS, anti-thymocyte serum; HIS, heat-inactivated serum; ATF3, activating transcription factor 3; Gadd45α, growth arrest and DNA damage-45 alpha; KLF6, Krüppel-like factor 6; PCNA, proliferating cell nuclear antigen; Dox, doxycycline; ChIP, chromatin immunoprecipitation.

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Mungre et al. 2009; Wang et al. 2009; Yin et al. 2008). However, no target genes of ATF3 in sublytic C5b-9-induced GMCs apoptosis have been identified. Therefore, the purpose of this study was to explore the mechanism involved in rat GMCs apoptosis, including identifying downstream target genes of ATF3 regulation in the GMCs apoptosis attacked by sublytic C5b-9.

Materials and methods

Reagents

Polyclonal antibodies against ATF3 (sc-188), Gadd45α (sc-6850), KLF6 (sc-20085) and β-actin (sc-77788) were purchased from Santa Cruz Biotechnology (USA), monoclonal antibody against PCNA was from Thermo scientific company (USA). PrimeScript™ RT reagent kit and SYBR Premix Ex Taq™ from Takara (Japan). Annexin V/PI apoptosis detection kit was from Biouniquer Technology Co., Ltd. (USA). Luciferase assay kits were from Promega (USA). ChIP analysis kits were from Upstate (Charlottesville, VA). Plasmids Tet-off advanced, TRE-tight-ATF3 and doxycycline (Dox) were from Clontech (USA).

The rat glomerular mesangial cell line (HBZY-1) was purchased from China Centre for Type Culture Collection (Wuhan, China). Normal human serum (NS) from healthy adult donors were pooled and used as a source of serum complement, and heat-inactivated serum (HIS) was prepared by incubating at 56°C for 30 min. Human complement C6-deficient serum (C6ds) was purchased from Sigma (USA). Rabbit polyclonal antibody against Thy-1 antigen of rat thymocyte, namely the anti-thymocyte serum (ATS), was prepared according to previously published procedures (Gao et al. 2006; Qiu et al. 2009; Cybulsky et al. 2005; Bosnich et al. 2005). The rat glomerular mesangial cell line (HBZY-1) was purchased from China Centre for Type Culture Collection (Wuhan, China). Normal human serum (NS) from healthy adult donors were pooled and used as a source of serum complement, and heat-inactivated serum (HIS) was prepared by incubating at 56°C for 30 min. Human complement C6-deficient serum (C6ds) was purchased from Sigma (USA). Rabbit polyclonal antibody against Thy-1 antigen of rat thymocyte, namely the anti-thymocyte serum (ATS), was prepared according to previously published procedures (Gao et al. 2006; Qiu et al. 2009; Xu et al. 2006). Ad-Max™-GFP, adenovirus recombinant named Ad-Tet-off and Ad-TRE-tight-ATF3, Ad-vec (Ad-TRE-tight-ATF3), Ad-ATF3 (Ad-Tet off + Ad-TRE-tight-ATF3), Dox (Ad-Tet off + Ad-TRE-tight-ATF3 plus 1 μg/mL Dox), sublytic C5b-9, Ad-vec + sublytic C5b-9, Ad-ATF3 + sublytic C5b-9, Dox + sublytic C5b-9. Moreover, ATF3 ShRNA expression plasmids were transiently transfected into the cultured GMCs, and the ShRNA that could effectively silence the ATF3 gene was chosen as our study mentioned previously (Jiang et al. 2010).

GMCs culture and sublytic C5b-9 determination

Rat GMCs were cultured at 37°C, 5% CO₂ conditions and maintained in MEM supplemented with 10% FBS, and used from five to seven passages for experiments. The formation of sublytic C5b-9 on GMCs membrane was identified as previously described (Gao et al. 2006, 2009; Qiu et al. 2009). The selection of ATS and complement concentration used in this study was 4% ATS and 4% NS with less than 5% LDH release or cell lysis. To demonstrate that the effects on GMCs following above stimulation were in fact due to sublytic C5b-9, control GMCs were treated with MEM, 4% ATS, 4% ATS + 4% HIS and 4% ATS + 4% C6ds (Gao et al. 2006; Qiu et al. 2009; Cybulsky et al. 2005; Bosnich et al. 2005).

GMCs infection, transfection and identification

An effective AdMax™-Tet off advanced system to regulate ATF3 expression in the GMCs was established, and the cultured GMCs were infected with this system for the indicated time (4 h, 12 h, 24 h and 48 h) or different doses of Dox to demonstrate the role of ATF3 in the GMCs. To further investigate the role of ATF3 in the GMCs induced by sublytic C5b-9, the cultured GMCs were infected with AdMax™-Tet-off advanced system for 42 h, then attacked by sublytic C5b-9 for 6 h. Experimental groups were as follows: MEM, Ad-vec (Ad-TRE-tight-ATF3), Ad-ATF3 (Ad-Tet off + Ad-TRE-tight-ATF3), Dox (Ad-Tet off + Ad-TRE-tight-ATF3 plus 1 μg/mL Dox), sublytic C5b-9, Ad-vec + sublytic C5b-9, Ad-ATF3 + sublytic C5b-9, Dox + sublytic C5b-9. Moreover, ATF3 ShRNA expression plasmids were transiently transfected into the cultured GMCs, and the ShRNA that could effectively silence the ATF3 gene was chosen as our study mentioned previously (Jiang et al. 2010).

Besides, three classes of SiRNA to different target sites of Gadd45α or KLF6 were designed (Table S2). And SiGadd45α and SiKLF6 were transiently transfected into the cultured rat GMCs with RNAi-Mate according to the manufacturer’s instructions, respectively, subsequently treated with sublytic C5b-9 for 6 h. The optimal target sequence to silence Gadd45α or KLF6 was selected (Fig. S1).

Real-time PCR analysis

The quantification of ATF3, Gadd45α, KLF6 and PCNA expression in the fixed time was performed using real-time PCR through ABI PRISM 7300 sequence detection system. Rat GAPDH was co-amplified as an internal control. The primers were listed in Table S3. The relative level of 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 GAPDH using the following formula: \[ \frac{2^{\Delta \text{CT} \text{in control sample} - \Delta \text{CT} \text{in stimulated sample}}} \]

Western blot analysis

The cultured GMCs were homogenized with lysis buffer. Proteins from each sample were assayed by SDS–PAGE using a 15% SDS–PAGE gel, then were transferred to PVDF membranes and probed with anti-ATF3 (diluted 1:800), anti-Gadd45α (diluted 1:600), anti-KLF6 (diluted 1:600) or anti-PCNA (diluted 1:1000).
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Fig. 2. Regulation of Gadd45α, KLF6 and PCNA protein levels in ATF3-expressing GMCs. (A) Photomicrographs of ATF3, Gadd45α, KLF6 and PCNA protein expression in the GMCs infected with AdMax™-Tet off system for the indicated time by Western blot. Semi-quantitative analysis showed that ATF3, Gadd45α and KLF6 protein levels in the GMCs infected with Ad-ATF3 were markedly increased at 24 h and 48 h (*P < 0.05, **P < 0.01), compared with Ad-vec group for the same treatment, and PCNA protein level displayed no statistic significance between two groups. Ad-vec represented for Ad-Tet off or Ad-TRE-tight-ATF3, Ad-ATF3 represented for Ad-Tet off + Ad-TRE-tight-ATF3.

(B) ATF3, Gadd45α, KLF6 and PCNA protein expression in the GMCs infected with AdMax™-Tet off system and treated with different doses of Dox for 48 h by Western blot. Semi-quantitative analysis showed that ATF3, Gadd45α and KLF6 protein levels were decreased gradually with increasing doses of Dox, compared with AdMax™-Tet

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antibodies. The bands were visualized by the ECL detection system with 10 min exposure. Immunoreactive band density was measured using Quantity One 1-D analysis software (BioRad, USA). The level of ATF3, Gadd45α, KL6 and PCNA protein in each group was expressed relative to the control groups.

**FACS detection**

Approximately 5 × 10⁵ GMCs were washed with PBS, and resuspended in binding buffer containing AnnexinV/PI. The samples were analyzed on a FACScan flow cytometer (BectonDickinson, SanJose, USA). The percentage of apoptotic cells in a 10,000-cell cohort was determined by flow cytometry. Each sample was assayed in triplicate.

**Luciferase reporter assay**

The cultured GMCs were seeded in 6-well plates at a density of 3 × 10⁴ per well over night, and were transfected with 4 μg of a luciferase reporter (pGL3 reconstructed with different regions of Gadd45α or KL6 promoter) and 4 μg PRL-SV40, using *GenEscort™ III* according to the manufacturer’s instructions. Then the cultured GMCs were treated with sublytic C5b-9 for 6 h or infected with AdMax™-tet off system for 48 h. The cells were then lysed and 20 μL of lysates assayed for luciferase activity using the dual luciferase reporter assay system (Promega) in a Monolight 2010 luminometer. Luciferase activity was normalized to PRL-SV40 activity to account for transfection efficiency and presented as relative luciferase units.

**Chromatin immunoprecipitation (ChIP) assay**

ChIP assays were performed as the protocol by Upstate. GMCs (2 × 10⁵ cells) were infected with AdMax™-Tet off system at m.o.i. 75 for 48 h, then GMCs were cross-linked with 1% formaldehyde for 10 min. After washing twice with PBS, the cells were collected and lysed with 2 ml of SDS lysis buffer containing a protease inhibitor mixture, and sonicated to DNA lengths of 200–500 bp. After centrifugation at 13,000 rpm for 10 min, supernatants were diluted 10 fold in ChIP dilution buffer. Immunoprecipitations were then performed with ATF3 antibody. ChIP DNA was detected using standard PCR with the following primer pairs for different regions of the Gadd45α and KL6 promoter, as listed in Table S4.

**Statistical analysis**

All data were summarized as mean ± SD. One-way ANOVA with simultaneous multiple comparisons between groups by the Bonferroni method was used, and *P* < 0.05 was considered significant. All experiments were performed in triplicate.

**Results**

**Expression of ATF3, Gadd45α, KL6 and PCNA in the GMCs attacked by sublytic C5b-9**

To investigate the expression of pathogenic genes in the GMCs attacked by sublytic C5b-9, microarray experiments were performed, and results revealed that different kinds of pathogenic genes, especially ATF3, Gadd45α, KL6 and PCNA in cultured primary rat GMCs attacked by sublytic C5b-9 in *vitro* at 40 min and 3 h were significantly up-regulated (data not shown). Then, we selected ATF3, Gadd45α, KL6 and PCNA gene to test their mRNA and protein levels in the cultured rat GMCs after sublytic C5b-9 stimulation. It was found that mRNA levels of ATF3, Gadd45α and KL6 in the GMCs induced by sublytic C5b-9 were up-regulated at 40 min, significantly increased at 3 h, then decreased at 6 h, and PCNA mRNA expression was up-regulated slightly at 3 h and 6 h (Fig. 1A). However, ATF3 protein expression in the GMCs in response to sublytic C5b-9 attack emerged at 3 h, markedly increased at 6 h, and gradually decreased at 12 h and 24 h. In addition, KL6 and Gadd45α protein expression became significantly increased at 3 h and 6 h, and gradually decreased at 12 h and 24 h, but PCNA protein expression elevated at 6 h and 12 h, and reached a peak at 24 h (Fig. 1B).

To avoid the effects of MEM, ATS or human serum and determine that C6 is necessary for C5b-9 assembly on the membrane of GMCs, the cultured GMCs treated for 6 h were analyzed, and the results showed that expression of ATF3, Gadd45α and KL6 in sublytic C5b-9 group was significantly higher than those in MEM, ATS, ATS + HIS and ATS + C6ds group, and PCNA expression was slightly increased in sublytic C5b-9 group (Fig. 1C and D). Taken together, the up-regulation of ATF3, Gadd45α, KL6, and PCNA was due to sublytic C5b-9 attack. Moreover, GMCs apoptosis induced by sublytic C5b-9 was again demonstrated by FACS (Fig. S2).

**Regulation of Gadd45α, KL6 and PCNA gene in the GMCs expressed ATF3**

To explore the role of ATF3 in the signaling cascade that connects sublytic C5b-9 attack and expression of Gadd45α, KL6 and PCNA, an AdMax™-Tet-off system to regulate ATF3 expression by different doses of Dox was established (Fig. S3A and B). Using this system, the expression of Gadd45α and KL6 in ATF3-expressing GMCs was up-regulated, but PCNA expression was not altered (Fig. 2A). When ATF3 expression was gradually decreased in the GMCs via increasing dose of Dox, the expression of Gadd45α and KL6 was gradually reduced, but PCNA expression was not changed (Fig. 2B). Additionally, ATF3 expression could indeed promote GMCs apoptosis (Fig. S3C).

To further confirm the effect of ATF3 on regulation of Gadd445α and KL6, the cultured GMCs were infected with AdMax™-Tet off system, and subsequently stimulated by sublytic C5b-9. The results displayed that ATF3 protein level in the GMCs treated with Ad-ATF3 + sublytic C5b-9 was significantly higher than other treatments, and Gadd45α and KL6 protein levels also markedly increased in the GMCs treated with Ad-ATF3 + sublytic C5b-9 than other treatments (Fig. 2C). Furthermore, the number of apoptotic GMCs in Ad-ATF3 + sublytic C5b-9 group was significantly higher than those in Ad-ATF3, sublytic C5b-9, Ad-vec + sublytic C5b-9, Dox + sublytic C5b-9 groups, although these groups displayed increased GMCs apoptosis compared with MEM, Ad-vec or Dox group (Fig. S4A). Changes of Gadd45α, KL6 and PCNA protein levels after sublytic C5b-9 attack were also monitored in the GMCs off system alone (*P* < 0.05, **P** < 0.01), but PCNA expression showed no statistical significance in each group. (C) ATF3, Gadd45α, and KL6 protein expression in the GMCs treated with AdMax™-Tet off system and sublytic C5b-9 complexes by Western blot. ATF3 over-expressing GMCs and groups attacked by sublytic C5b-9 displayed significantly up-regulations of the expression of ATF3, Gadd45α, and KL6 compared with MEM. Ad-vec or Dox group (*P* < 0.05, **P** < 0.01). Furthermore, in Ad-ATF3 over-expressing GMCs (Ad-Tet off + Ad-TRE-tight-ATF3) + sublytic C5b-9 was significantly higher than those in Ad-TATF3 over-expressing GMCs or sublytic C5b-9 attack alone (*P* < 0.001). (D) ATF3, Gadd45α, KL6, and PCNA protein expression in the GMCs treated with ShATF3. The protein levels of ATF3, Gadd445α, and KL6 in ShATF3 + sublytic C5b-9 group were significantly lower than those in the ShControl + sublytic C5b-9 group (*P* < 0.05). β-Actin was used as control for protein level. All data represent as mean ± SD from three independent experiments.

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transfected with ATF3 shRNA. In the GMCs transfected with ShATF3 and treated with sublytic C5b-9 for 6 h, the ATF3 induction was nearly completely ablated. Both Gadd45α and KLF6 protein levels were decreased and maintained a lower level in the GMCs transfected with ShATF3, but not PCNA protein level (Fig. 2D). Moreover, our results also found that the number of apoptotic GMCs in ShATF3 + sublytic C5b-9 group was significantly lower than that in ShControl + sublytic C5b-9 group (Fig. 5B). The results indicated that both endogenous and exogenous expression of ATF3 in the GMCs could up-regulate expression of Gadd45α and KLF6 gene, and ATF3 was involved in promoting GMCs apoptosis induced by sublytic C5b-9 attack.

Direct target genes of ATF3 in the GMCs induced by sublytic C5b-9

Although the results mentioned-above showed an altered expression of Gadd45α and KLF6 in ATF3-expressing GMCs, they did not indicate whether Gadd45α and KLF6 were direct target genes of ATF3 or not. To address this issue, Gadd45α and KLF6 gene promoter sequences were analyzed using the MotifScanner. Both Gadd45α and KLF6 contained putative binding sites for CREB motifs: Gadd45α at position −122/−133, and KLF6 at position −198/−209 relative to the transcriptional start site, respectively. However, for some promoters, the sites are more than 2 kilobases (kb) upstream from the transcriptional start (+1) site, but the potential binding site within 250 base pair (bp) from the +1 site was focused in our experiments.

In order to elucidate the effect of ATF3 on Gadd45α gene transcription directly, luciferase assay was performed. Compared with control treatments, significant enhancement of luciferase activity was observed in the GMCs transfected with Gadd45α promoter including predicted CREB site, in which ATF3 expression was induced by sublytic C5b-9, but there was no statistical significance of luciferase activity in the cells transfected with Gadd45α promoter without the predicted CREB site (Fig. 3A). These results were consistent with those in ATF3-expressing GMCs infected with AdMaxTM−Tet off system (Fig. 3B), suggesting that ATF3 could activate the Gadd45α promoter. Next, to identify whether ATF3 binds to the predicted CREB site of Gadd45α or not, ChIP analysis was also performed, cross-linked and sheared chromatin was immunoprecipitated with antibody against ATF3. The specificity of the band was demonstrated by lack of signals when control immunoglobulin (IgG) or 1 μg/ml Dox. The precipitated DNA was amplified with primers encompassing the proximal region of Gadd45α (Fig. 3C). Over-expression of ATF3 was shown in −Dox group (Fig. 3D), which resulted in a ChIP signal delivered from two different primers flanking the CREB-like binding site on the Gadd45α promoter, and no ChIP signal was detected in +Dox group (Fig. 3E). Taken all together, these results revealed that Gadd45α is a bona fide target gene of ATF3 in rat GMCs.

To demonstrate the effect of ATF3 on KLF6 gene transcription, luciferase assay was also done. However, whether KLF6 promoter including the predicted CREB site or not, no significant difference of luciferase activity was found in all treated GMCs (Fig. 4A), which was consistent with that in ATF3-expressing GMCs infected with AdMaxTM−Tet off system (Fig. 4B). Furthermore, ChIP analysis was performed to identify the binding ability of ATF3 to the predicted CREB site of KLF6 promoter (Fig. 4C). Over-expression of ATF3 was found in −Dox group, but the results did not show a significant ChIP signal derived from two different primers flanking the CREB-like binding site on KLF6 promoter, neither in −Dox group nor in +Dox group (Figs. 3D and 4D). These results suggested that ATF3, which regulated KLF6 gene expression, might be dependent upon an indirect manner.

Role of Gadd45α or KLF6 in sublytic C5b-9-induced GMCs apoptosis

To further assess the role of Gadd45α in GMCs apoptosis mediated by sublytic C5b-9, GMCs were transfected with SiGadd45α for 16 h to knock down the expression of Gadd45α gene. After the GMCs stimulated by sublytic C5b-9 for 6 h, the protein level of Gadd45α was determined by Western blot. The results manifested that the level of Gadd45α protein in the GMCs transfected with SiGadd45α following sublytic C5b-9 stimulation was significantly decreased in comparison with SiControl + sublytic C5b-9 (Fig. 5A). And GMCs apoptosis was also significantly decreased in SiGadd45α + sublytic C5b-9 group, which indicated that Gadd45α gene knockdown in the GMCs could notably suppress GMCs apoptosis induced by sublytic C5b-9 attack (Fig. 5B). Interestingly, the expression of ATF3, KLF6 and PCNA was not altered when Gadd45α expression in the GMCs was silenced (Fig. 5A).

To evaluate the effect of KLF6 on GMCs apoptosis, GMCs were transfected with SiKLF6 to knock down the expression of KLF6. The results demonstrated that KLF6 expression was not only silenced successfully in SiKLF6 + sublytic C5b-9 group (Fig. 5C), but also GMCs apoptosis was decreased, indicating that KLF6 gene knockdown in the GMCs could also markedly inhibit GMCs apoptosis triggered by sublytic C5b-9 (Fig. 5D). However, when KLF6 gene expression was silenced, the expression of ATF3, Gadd45α and PCNA gene was not altered (Fig. 5C).

Discussion

Early studies reported that in the early phase of Thy-1N model, GMCs are targeted by anti-Thy-1 antibody resulting in immediate complement activation and cell injury (Brandt et al. 1996), and GMCs apoptosis/necrosis is considered to be a contributor to the initiation of Thy-1 nephritis (Amore and Coppo 2000; Qiu et al. 2009; Shimizu et al. 2000; Xu et al. 2006; Yamamoto and Wilson 1987). In this study, we confirmed that sublytic C5b-9 complexes could indeed lead to GMCs apoptosis. Although we had implicated that GMCs apoptosis induced by sublytic C5b-9 in rats with Thy-1N was dependent on Gadd45α up-regulation in part (Qiu et al. 2009), the molecular mechanisms responsible for GMCs apoptosis of Thy-1N, especially sublytic C5b-9-induced GMCs apoptosis, have not been well elucidated.

It is well known that ATF3 could play a critical role in mediating cellular apoptosis or proliferation, and the diversity in the final readouts is most likely to be determined by the context of cells (Chen et al. 2008; James et al. 2006; Mungreue et al. 2009). In our experiments, we found that ATF3 mRNA and protein levels in the GMCs attacked by sublytic C5b-9 were remarkably increased, and ATF3 expression could indeed promote GMCs apoptosis triggered by sublytic C5b-9 attack. And our results also showed that Gadd45α and KLF6 were significantly up-regulated and co-expressed with ATF3. In order to explore the mechanism of GMCs apoptosis mediated by sublytic C5b-9 stimulation and to identify downstream target genes of up-regulated ATF3 in the GMCs exposed to sublytic C5b-9, we analyzed Gadd45α and KLF6 promoter sequences using the MotifScanner, and found that both of them including the predicted ATF/CRE binding site.

As the most well known gene in the Gadd45 family (including Gadd45α, Gadd45β, Gadd45γ), Gadd45α is a small acidic protein consisting of 165 amino acids and is preferentially localized in the nuclei. Indeed, the majority of the known Gadd45α interacting molecules are nuclear functional proteins, such as p21[cyclin-dependent kinase inhibitor 1A], xeroderma pigmentosum complementation group G and so on (Barreto et al. 2007; Kearsey et al. 1995). Interaction of Gadd45α with these proteins causes cell cycle arrest, repression of DNA replication or...
Fig. 3. Activity analysis of Gadd45α gene promoter and binding of ATF3 to Gadd45α gene promoter in ATF3-expressing GMCs. (A) The cultured rat GMCs were transiently transfected with equal amounts of Gadd45α promoter constructs (pGL3-basic-Gadd45α-1 and pGL3-basic-Gadd45α-2 included the potential ATF/CRE binding sites, pGL3-basic-Gadd45α-3 did not include) and PRL-SV40 for the indicated time, then treated with sublytic C5b-9 for 6 h, then the cells were assayed for luciferase activity. Significant enhancement of luciferase activity was observed in the ATF3-expressing GMCs induced by sublytic C5b-9, which transfected with the pGL3-basic-Gadd45α-1 or pGL3-basic-Gadd45α-2 (*P < 0.01 vs. MEM, □P < 0.01 vs. ATS, ■P < 0.01 vs. ATS + His, #P < 0.01 vs. ATS + C6dS), but not transfected with pGL3-basic-Gadd45α-3. (B) The cultured rat GMCs were co-transfected with Gadd45α promoter constructs and PRL-SV40 for the indicated time, then infected with AdMaxTM-Tet off system. Significant increase of luciferase activity was observed in the −Dox group (Ad-Tet off + Ad-TRE-tight-ATF3), which transfected with pGL3-basic-Gadd45α-1 or pGL3-basic-Gadd45α-2 (*P < 0.01 vs. Ad-Tet off + Ad-TRE-tight-ATF3 plus 1 μg/mL Dox), but not transfected with the pGL3-basic-Gadd45α-3. (C) Schematic of Gadd45α: the transcriptional start (+1), potential ATF/CRE binding sites and the primer target sites were indicated. (D and E) The cultured GMCs were infected with Ad-Tet off + Ad-TRE-tight-ATF3 or Ad-Tet off + Ad-TRE-tight-ATF3 plus 1 μg/mL Dox for 48 h. Immunoprecipitated DNA was amplified by PCR for the proximal promoter regions of Gadd45α gene. The results displayed that ATF3 protein level in ATF3-expressing GMCs was significantly higher than that in 1 μg/mL Dox treated GMCs (*P < 0.01), and there was a significant ChIP signal in the ATF3-expressing GMCs, derived from two different primers flanking the CREB-like binding site on Gadd45α promoter.
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Fig. 4. Activity analysis of KLF6 gene promoter and binding of ATF3 to KLF6 gene promoter in ATF3-expressing GMCs. (A and B) The cultured rat GMCs were transiently transfected with equal amounts of KLF6 promoter constructs (pGL3-basic-KLF6-1 included potential ATF/CRE binding sites, pGL3-basic-KLF6-2 did not include) and PRL-SV40 for the indicated time, and then treated with sublytic C5b-9 complexes or infected with AdMaxTM-Tet off system. The cells were assayed for luciferase activity. No significant enhancement of luciferase activity was observed in neither sublytic C5b-9-induced nor AdMaxTM-Tet off system regulated ATF3-expressing GMCs, which transfected with pGL3-basic-KLF6-1 or pGL3-basic-KLF6-2. Values were mean ± SD and expressed as fold increase relative to similarly transfected controls. (C) Schematic of KLF6: the transcriptional start (+1), potential ATF/CRE binding sites and the primer target sites were indicated. (D) The cultured GMCs were infected with AdMaxTM-Tet off system for 48 h. Cells were harvested for ATF3 protein expression, and ChIP assay was performed using anti-ATF3 antibody or control IgG. Immunoprecipitated DNA was amplified by PCR for the proximal promoter regions of KLF6 gene. The results showed that ATF3 protein level in Ad-Tet off + Ad-TRE-tight-ATF3 group was significantly higher than that in the 1 μg/mL Dox treated GMCs (P < 0.01), but ATF3 did not bind directly to KLF6 promoter. All data were from three independent experiments.

KLF6, another potential downstream target gene of ATF3, is a zinc-finger nuclear protein that belongs to the Krüppel-like family of transcription factors. KLF6 contains three C2H2 zinc fingers at the C-terminal domain, recognizes GC or CACCC motifs, and is broadly expressed in numerous cell types at various developmental stages (Slavin et al. 1999). KLF6 activation can result in cell cycle arrest, ratio of GMCs in response to sublytic C5b-9 attack decreased significantly when Gadd45α gene was silenced. These results indicated that Gadd45α gene might be downstream target gene of ATF3. In other words, ATF3 could play an important role in promoting GMCs apoptosis triggered by sublytic C5b-9 complexes directly through up-regulation of Gadd45α gene.
Fig. 5. Effects of Gadd45α or KLF6 on sublytic C5b-9-induced GMCs apoptosis. (A) Numbers of apoptotic GMCs treated by SiGadd45α were analyzed by flow cytometry. The number of apoptotic GMCs in SiGadd45α + sublytic C5b-9 group was significantly lower than that in SiControl + sublytic C5b-9 group (p < 0.05). (B) ATF3, Gadd45α, KLF6 and PCNA protein expression in the GMCs treated by SiGadd45α. The protein level of Gadd45α in the SiGadd45α + sublytic C5b-9 group was significantly lower than that in the SiControl + sublytic C5b-9 group (p < 0.05), but protein level of ATF3, KLF6 and PCNA showed no statistical significance between two groups. (C) Numbers of apoptotic GMCs treated with SiKLF6 were analyzed by flow cytometry. The number of apoptotic GMCs in SiKLF6 + sublytic C5b-9 group was significantly lower than that in SiControl + sublytic C5b-9 group (p < 0.05). (D) ATF3, Gadd45α, KLF6 and PCNA protein expression in the GMCs treated with SiKLF6. The protein levels of KLF6 in SiKLF6 + sublytic C5b-9 group were significantly lower than that in SiControl + sublytic C5b-9 group (p < 0.05), but protein level of ATF3, Gadd45α and PCNA showed no statistical significance between two groups.
decreased angiogenesis and invasion. Recent study also demonstrates that the role of transfected exogenous KLF6 in suppressing tumor growth of lung cancer cells is due to cell apoptosis (Ito et al. 2004). As a transcription factor, KLF6 targets include transforming growth factor (TGF)–β1, TGF-β receptor, urokinase plasminogen activator, inducible nitric oxide synthase and so on (Kojima et al. 2000; Warke et al. 2003). However, little information is available regarding cellular factors involved in regulating KLF6 gene expression. In our experiment, we revealed that either endogenous or exogenous expression of ATF3 could up-regulate KLF6 gene expression in the GMCs exposed to sublytic C5b-9. However, our further results suggested that ATF3 over-expression in rat GMCs could not increase KLF6 promoter activity, and could not bind to the predicted CREB site of KLF6 promoter. Moreover, GMCs apoptosis induced by sublytic C5b-9 decreased significantly when KLF6 was silenced. Taken together, ATF3 promoted sublytic C5b-9-mediated GMCs apoptosis might be due to up-regulation of KLF6 in an indirect manner. Here, it was worthy to note that our results were inconsistent with Huang’s data, in which ATF3 is a key target gene of KLF6 in tumor suppression, and ATF3 can mediate KLF6-induced cancer cells apoptosis under stress conditions (Huang et al. 2008). Based on our data, we thought that the different results might associate with initiating distinct signal pathways under different stimulations and different cell types.

In addition, PCNA, which plays a central role in cell cycle regulation, was also up-regulated in sublytic C5b-9-induced GMCs. Previous studies have demonstrated that ATF3 directly regulates the PCNA-associated factor KIAA0101/p15PAF (Turchi et al. 2009). Using the MotifScanner, we found that PCNA also contained putative binding sites for CREB at position 189–199 relative to the transcriptional start site. But our further experiments showed that ATF3 expression in rat GMCs did not regulate PCNA expression, and not bind to the predicted CREB site of PCNA promoter. As PCNA reached a peak level in the GMCs treated with sublytic C5b-9 later at 24 h, we considered that PCNA expression might be related to GMCs secondary proliferation. Interestingly, some reports indicate that Gadd45α appears to interact with PCNA through its flexible loop (Sánchez et al. 2010; Vairapandi et al. 2000), which suggests a role of Gadd45α gene might be involved in regulating PCNA function, but not PCNA expression.

In conclusion, our results indicated that increase of ATF3 expression had a promoting role in GMCs apoptosis triggered by sublytic C5b-9 attack, and the mechanism of ATF3 increasing GMCs apoptosis in response to sublytic C5b-9 was associated with direct up-regulation of Gadd45α gene expression and indirect up-regulation of KLF6 gene expression.

Conflict of interest

The authors declare that there is no duality of interest associated with this manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.imbio.2011.02.005.
that includes ATM nibrin1, stress–induced MAP kinases and ATF-2. Oncogene 22, 4235–4242.


