Effects of PI3-k/Akt short hairpin RNA on proliferation, fibronectin production and synthesis of thrombospondin-1 and transforming growth factor- β_1 in glomerular mesangial cells induced by sublytic C5b-9 complexes

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Received 7 March 2008; revision accepted 7 April 2008

Abstract

Objectives: To explore proliferation of glomerular mesangial cells (GMC) and secretion of extracellular matrix (fibronectin induced by sublytic C5b-9 complexes), and then ascertain the role of phosphatidylinositol 3-kinase (PI3-k)/Akt signal pathway in these processes, by using small hairpin RNAs.

Material and methods: The expression of cyclin D₂, ³H-thymidine into DNA and production of fibronectin including thrombospondin-1 and transforming growth factor- β_1 in the GMCs stimulated by sublytic C5b-9 or transfected with expression vectors of PI3-k and Akt short hairpin RNA or LY294002 (PI3-k inhibitor) were measured by Real-time quantitative polymerase chain reaction (PCR), Western blot, enzyme-linked immunosorbent assay (ELISA) and ³H-thymidine incorporation (³H-TdR), respectively.

Results: The expression of cyclin D₂, ³H-thymidine into DNA and fibronectin in the GMCs stimulated by sublytic C5b-9 could all be increased, and the elevations of these parameters mentioned above were also markedly reduced in the GMCs transfected with vectors of PI3-k and Akt short hairpin RNA or LY294002, respectively.

Conclusions: These data indicate that sublytic C5b-9 can promote proliferation of GMCs and secretion of fibronectin as well as synthesis of thrombospondin-1 and transforming growth factor- β_1 . The PI3-k/Akt

signal pathway in these reactions, mediated by sublytic C5b-9 complexes, may play at least a partial role.

Introduction

Proliferation of glomerular mesangial cells (GMC) and secretion of extracellular matrix (ECM) are common cellular responses to a variety of different types of glomerular injury in several animal and human nephritides (1,2). Considerable evidence has suggested that GMC proliferation could result in an increase of mesangial matrix and development of glomerulosclerosis (3–5). Rat anti-Thy 1 nephritis, namely, antithymocyte serum-induced nephritis, is a model for studying human mesangioproliferative glomerulonephritis. Early experiments on anti-Thy 1 nephritis demonstrated that injuries of glomerular cells, including lysis plus proliferation and ECM secretion, were complement-dependent and neutrophil independent (3,6,7). Sublytic quantities of complement C5b-9 complexes (sublytic C5b-9), which insert into the cell membrane, are a potent stimulus for cell activation, especially for cell proliferation. Previous reports have revealed that GMC damage mediated by sublytic C5b-9 was involved in some mechanisms, such as production of oxidants, proteases, nitric oxide and some cytokines such as thrombospondin-1 (Tsp-1) and transforming growth factor- β_1 (TGF- β_1) (3,7–9). Because the pathological changes of GMCs (cell proliferation and ECM production) in anti-Thy 1 nephritis are complement-dependent, the role of sublytic C5b-9 on proliferation of cells and secretion of ECM, by using the C5b-9-GMC model *in vitro* is worthy of further study (6,10).

Tsp-1 is a 450-kDa trimeric extracellular matrix glycoprotein, which is found in platelet β -granules and other cell types including GMCs. Many studies have indicated that Tsp-1 has diverse effects on cell behaviour (11–13); it not only promotes cell proliferation, but also mediates ECM secretion through activation of TGF- β_1 (14,15). TGF- β_1 is a key cytokine that causes tissue fibrosis in

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inflammatory disease by inducing ECM secretions in many cells types (16).

Akt (also called protein kinase B), the cellular homologue of the *v-akt* oncogene, is a 60-kDa serine/threonine kinase, cloned by virtue of its homology to protein kinase A (PKA) and protein kinase C (PKC). As is known, Akt is dependent on phosphatidylinositol 3-kinase (PI3-k) activity after binding to the pleckstrin homology domain of Akt, which permits association of the phosphatidylinositol-dependent kinases, PDK1, PDK2 and activation of Akt through phosphorylation of Thr³⁰⁸ and Ser⁴⁷³. Growing evidence indicates that PI3-k/Akt is a critical mediator of survival signals in multiple cell lines (17,18) and it has been studied extensively in regulation of cell population growth and survival (19–21). Although our previous studies have demonstrated that synthesis of Tsp-1 and TGF- β_1 induced by sublytic C5b-9 in GMCs was associated with activation of the PI3-k/Akt pathway (9), its role in sublytic C5b-9-induced GMC proliferation and ECM production has not yet been fully elucidated.

Small interfering RNA (siRNA), short hairpin RNA (shRNA), is now widely used to knockdown gene expression in a sequence-specific manner, making it a powerful tool for studying gene function (22–25). In the present study, we first determined the effects of sublytic C5b-9 on GMC proliferation and fibronectin production, and then observed the roles of PI3-k/Akt shRNA on GMC reactions including synthesis of Tsp-1 and TGF- β_1 by knocking down expression of the *PI3-k/Akt* gene.

Materials and Methods

Reagents

The rat GMC strain was provided by the China Centre for Type Culture Collection. shRNAs were synthesized by Wuhan Genesil Biotechnology Co., Ltd (Wuhan, China). Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA, USA). LY294002 (specific PI3-k inhibitor), mouse monoclonal antibody against phosphorylated Akt (P*-Akt), phototope-horseradish peroxidase (HRP) Western blot detection system, including anti-mouse IgG, HRP-linked antibody, biotinylated protein ladder, 20 $\times$ LumiGLO reagent and 20 $\times$ peroxide were purchased from Cell Signalling Technology (Beverly, MA, USA). Human complement C6-deficient serum (C6DS) was obtained from Sigma (St Louis, MO, USA). Mouse monoclonal antibodies against total Akt (T-Akt), cyclin D2, fibronectin, β -actin and goat polyclonal antibody against Tsp-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). M-MLV reverse transcriptase was obtained from Promega (Madison, WI, USA) and the rat activated TGF- β_1 ELISA kit was provided by Bio-source (Camarillo,

CA, USA). ³H-thymidine was donated by the isotope laboratory of Nanjing Medical University and Trizol reagent was purchased from Life Technology (Gaithersburg, MD, USA). Normal human sera, pooled from several healthy adult donors, were used as the source of serum complement. Heat-inactivated sera were obtained by incubating the normal human sera at 56 °C for 30 min. Rabbit polyclonal antibody (anti-Thy 1 antibody, titre 1:320) against Thy 1 antigen of rat thymocytes was prepared according to previous studies (7,26).

Culture of GMCs and dosage of sublytic C5b-9 stimulation

Rat GMCs were maintained in RPMI 1640 medium supplemented with 10% foetal calf serum (FCS), 100 U/ml penicillin, 100 U/ml streptomycin and 10% non-essential amino acids. Rat GMCs used in the experiments were sampled from five to seven passages. Selection of the anti-Thy 1 antibody and complement concentration used in this study was 5% anti-Thy 1 antibody and 4% normal human serum without producing GMC injury or lysis (9).

Generation of DNA sequence for transcription of 19 or 21 bp hairpin RNA of PI3-k shRNA and Akt shRNA

To ensure silencing of the objective genes, eight different target gene sequences of shRNA were designed to be homologous to PI3-k and Akt consensus sequences, respectively. The sequence of Pik3c3 from *Rattus norvegicus* phosphoinositide-3-kinase class 3 complete cds (GenBank accession number M022958) and the sequence of Pik3cb from the *R. norvegicus* phosphatidylinositol 3-kinase, catalytic subunit complete cds (GenBank accession number M053481) were chosen as shPI3-k target genes. The sequence of Akt from the *R. norvegicus v-akt* murine thymoma viral oncogene homolog 1 complete cds (GenBank accession number M033230) was chosen as shAkt target gene.

Construction of shPI3-k- and shAkt-expressing plasmid vector

Complementary oligonucleotides encoded a hairpin structure with a 19- or 21-mer stem, derived from the target site. A 9-bp loop sequence separated the two complementary domains. Near the 3' end of the shRNA template was a 6-nucleotide poly (T) tract recognized as an RNA pol III termination signal. The 5' end of the two oligonucleotides was *Bam*HI and *Hind*III restriction site overhung, for example, Akt1–1 DNA oligos. Vectors of shPI3-k- and shAkt-expressing plasmid were constructed by using pGenesil-1 as the vector backbone. These shRNAs were synthesized, annealed and ligated into the *Bam*HI and *Hind*III sites of linearized pGenesil-1 shRNA

expression vector. At the same time, an unrelated gene shRNA was chosen as a negative control.

Transfection of shRNA

Transient transfection of shRNA into the cultured GMCs was conducted using Lipofectamine 2000 according to the manufacturer's instructions. Briefly, 3.0 µg shRNA and 8.0 µl of the transfection medium were incubated for 25 min, then the mixture was added to the cultured GMCs. Culture medium was then removed at 6 h after transfection and replaced by minimum essential medium (MEM) supplemented with 10% FCS for 54 h. Green fluorescence protein was directly observed using a fluorescence microscope to evaluate the ratio of transfection.

Real-time quantitative polymerase chain reaction

Total RNA in different groups at fixed time was isolated by using Trizol. For Tsp-1, a fluorescence-labelled probe was used (Primer-F: 5'-CAG AAT GTG AGG TTT GTC TTT GGA-3', Primer-R: 5'-TTT GTT TTG TGG CCG ATG TAG T-3', FAM/TAMRA-labelled probe: 5'-TGC TCC AGT TCA ACC AAC GTC CTT CTC-3'). TGF-β₁ was quantified by using (Primer-F: 5'-AAG AAG TCA CCC GCG TGC TA-3', Primer-R: 5'-GGC ACT GCT TCC CGA ATG-3', FAM/TAMRA-labelled probe: 5'-TGG ACC GCA ACA ACG CAA TCT ATG A-3'). Cyclin D₂ was quantified by using (Primer-F: 5'-CAC CGA CAA CTC TGT GAA G-3', Primer-R: 5'-CAG AGC GAT GAA GGT CTG-3', FAM/TAMRA-labelled probe: 5'-CAG GAG CTG CTG GAG TGG GAA CT-3'). Fibronectin was quantified by using (Primer-F: 5'-TCA GAA CCG GAA CGG AGA AA-3', Primer-R: 5'-ACA TAC GTG AAT GCC AGT CCT TT-3', FAM/TAMRA-labelled probe: 5'-TGG TTC AGA CTG CAG TGA CCA ACA TTG A-3'). β-actin was quantified by using (Primer-F: 5'-TCA CCC ACA CTG TGC CCA TCT ATG A-3', Primer-R: 5'-CAT CGG AAC CGC TCA TTG CCG ATA G-3', FAM/TAMRA-labelled probe: 5'-ACG CGC TCC CCC ATG CCA TCC TGC GT-3'). Real-time polymerase chain reaction (PCR) was performed by ABI PRISM 7300 sequence detection system (Applied Biosystems, Foster City, CA, USA) with the thermal cycling conditions.

Western blot analysis

The lysate of the GMCs was centrifuged at 10 000 g for 10 min. Protein concentrations were determined using the Bio-Rad Protein Assay kit Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). Aliquots of 50 µg

protein were subjected to 12% and 8% sodium dodecyl sulphate-polyacrylamide gel electrophoresis, respectively. Thereafter, protein was blotted on to a polyvinylidene fluoride membrane. Primary antibodies against P*-Akt, T-Akt, Tsp-1, cyclin D2, fibronectin and β-actin were used according to the manufacturer's recommendations (respectively). A second antibody (HRP-conjugated anti-mouse or anti-goat IgG) was used for detection of the parameters mentioned above.

ELISA for TGF-β₁

Activated TGF-β₁ contents in the supernatants of cultured GMCs were detected according to ELISA kit indication. In brief, the cultured GMCs were co-incubated with different stimulations for 18 h. Then, 100-µl activated supernatants (diluted 20-fold) in the GMCs were added to a microplate precoated with TGF-β₁ monoclonal antibody. After incubation, diluted HRP-conjugated antibodies were added, and the colour intensity was measured using a microtitre plate reader at 450 nm.

Measurement of ³H-thymidine incorporation

Rat GMCs were plated into 96-well plates and incubated overnight. Media were removed from the cells and replaced with 200 µl media containing Thy 1 antibody and normal human serum to form sublytic C5b-9 complexes (9). The cells were incubated for 54 h, and DNA synthesis was determined by ³H-TdR incorporation for the final 18 h. Cells were harvested on to glass filters. Radioactivity retained on dried filters was measured and counted in a 1450 LSC & Luminescence Counter (Perkin Elmer, USA).

Statistical analysis

Data were expressed as mean ± standard error. Statistical evaluation of the data was performed by one-way analysis of variance, with simultaneous multiple comparisons between groups, by the Scheffe method, considering a *P*-value of < 0.05 as significant.

Results

Proliferation of GMCs and secretion of ECM induced by sublytic C5b-9

As shown in Fig. 1, GMCs attacked by sublytic C5b-9 could proliferate and secrete ECM. The level of cyclin D2 expression (mRNA and protein) induced by sublytic C5b-9 complexes, significantly increased in comparison to MEM and other treatment groups (Fig. 1a–b). There

Figure 1. Proliferation of glomerular mesangial cells (GMC) and secretion of extracellular matrix (ECM) were assessed by measuring cyclin D2 (a, b), the value of counts per minute (c) and fibronectin (FN) (d, e). Cultured GMCs were treated with sublytic C5b-9, anti-Thy 1 antibody (Ab), anti-Thy 1 Ab + heat-inactivated sera (HIS), anti-Thy 1 Ab + C6-deficient serum (C6DS) and MEM for 24 h. Expression of cyclin D2 and FN (mRNA and protein) was analysed by real-time polymerase chain reaction and Western blot analysis. β -actin was used as control. Data were expressed by mean $\pm$ standard error of three independent experiments (** $P < 0.01$ vs. MEM group; $\blacktriangle\blacktriangle P < 0.01$ vs. anti-Thy 1 Ab + C6DS group; $\#\#\# P < 0.01$ vs. anti-Thy 1 Ab + HIS group; $\times\times\times P < 0.01$ vs. anti-Thy 1 Ab group).

was a twofold increase in DNA synthesis in the GMCs exposed to sublytic C5b-9 for 24 h (Fig. 1c). In addition, the level of fibronectin expression in the cells stimulated by sublytic C5b-9 was also obviously increased compared to the MEM and other groups (Fig. 1d-e).

Activity of PI3-k/Akt induced by sublytic C5b-9

Rat GMCs were treated in dissimilar media: sublytic C5b-9, LY294002 + sublytic C5b-9, anti-Thy 1 antibody + heat-inactivated sera, anti-Thy 1 antibody + C6DS and MEM

Figure 2. Activation of phosphatidylinositol 3-kinase (PI3-k)/Akt in the cultured glomerular mesangial cells (GMC) induced by sublytic C5b-9 was observed. Rat GMCs were treated with sublytic C5b-9, LY294002 + sublytic C5b-9, anti-Thy 1 antibody (Ab) + heat-inactivated sera (HIS), anti-Thy 1 Ab + C6-deficient serum (C6DS) and MEM alone. Cells were lysed and examined for P*-Akt by Western blot analysis. The different activity of PI3-k was determined as described. The significant increase of P*-Akt was seen in the GMCs with sublytic C5b-9 stimulation. Graph shows relative phosphorylated Akt (P*-Akt) protein level normalized to total Akt (T-Akt). Results were expressed as mean $\pm$ standard error of three separate experiments (** $P < 0.01$ vs. MEM group; $\Delta P < 0.05$ vs. anti-Thy 1 Ab + C6DS group; $\#P < 0.05$ vs. anti-Thy 1 Ab + HIS group; $^{xx}P < 0.01$ vs. LY294002 + sublytic C5b-9 group).

alone. Akt activation by sublytic C5b-9 was determined by *in vitro* kinase assay and by assessing P*-Akt at Ser⁴⁷³. As shown in Fig. 2, Akt phosphorylation at Ser⁴⁷³ reached a maximum when GMCs were stimulated by sublytic C5b-9, as compared with LY294002 + sublytic C5b-9, anti-Thy 1 antibody + heat-inactivated serum and anti-Thy 1 antibody + C6DS groups. Inhibition of PI3-k with LY294002 could abolish the stimulatory effect of sublytic C5b-9 on Akt and GMCs sensitized by anti-Thy1 antibody were incubated with heat-inactivated serum, C6DS or normal human serum (namely, the sublytic C5b-9 group). Akt phosphorylation (P*-Akt) markedly increased with normal human serum treatment only, whereas P*-Akt did not show any obvious change in anti-Thy 1 antibody + heat-inactivated serum and anti-Thy 1 antibody + C6DS. These results suggest that the PI3-k/Akt pathway stimulated by sublytic C5b-9 can be activated.

Identification and evaluation of shRNA expression plasmids

Expression plasmids of shPik3c3-2, shAkt1-4 and shGAPDH were digested by restriction enzyme *SalI*, and electrophoresis in 1% agarose gel produced fragments of 405 bp and 4.9 kb. Sequence analysis confirmed that the expression plasmids of pGenesil-1 shRNA were correct. In the experiment, expression vectors of shGAPDH or negative shRNA were transfected into GMCs at different times. Efficiency of transfection could be observed by fluorescence of green fluorescence protein, which reached a maximum at 54 h after shRNA transfection.

The cultured GMCs were transfected with eight pairs of shRNA to repress endogenous PI3-k and Akt target genes, respectively. Levels of P*-Akt and T-Akt protein in the GMCs were measured by using Western blot analysis and expression of P*-Akt and T-Akt were found to be significantly lower after transfection with the most efficient shRNA in a defined time. However, in other invalid shRNA groups, P*-Akt and T-Akt proteins showed no apparent change. As shown in Fig. 3(a,b), the optimal shRNA vectors that could effectively silence the target genes were shPik3c3-2 and shAkt1-4.

In order to assess the specificity of shPik3c3-2 and shAkt1-4, the roles of shPik3c3-2 and shAkt1-4 on the expression of P*-Akt and T-Akt were examined separately. As shown in Fig. 3(c), shPik3c3-2 and shAkt1-4 could decrease production of P*-Akt and T-Akt in a dose-dependent manner. When the GMCs were transfected with serially increasing doses of shRNA, P*-Akt and T-Akt were markedly reduced. These results revealed that shPik3c3-2 and shAkt1-4 were very effective in silencing *PI3-k* and *Akt* genes.

Effects of shPik3c3-2 and shAkt1-4 on GMC proliferation and fibronectin production induced by sublytic C5b-9

The results described above demonstrated that shPik3c3-2 and shAkt1-4 could down-regulate expression of P*-Akt and T-Akt, respectively. At the same time, changes of cyclin D2, GMC proliferation and fibronectin production were also examined. The data showed that the parameters (levels of cyclin D2, GMC proliferation and fibronectin production) in shPik3c3-2 + sublytic C5b-9, shAkt1-4 + shPik3c3-2 + sublytic C5b-9 groups exhibited a significant decrease and a slight decrease in the shAkt1-4 + sublytic C5b-9 group; there was no decrease in the negative shRNA group (Fig. 4). These results indicate that inhibition of *PI3-k* and *Akt* gene expression could reduce GMC proliferation and fibronectin secretion, but it was not sufficient to completely inhibit an increase in GMC proliferation and fibronectin secretion in the GMCs

Figure 3. Phosphorylated Akt (P*-Akt) and total Akt (T-Akt) in the glomerular mesangial cells (GMC) treated with short hairpin RNA (shRNA) for phosphatidylinositol 3-kinase (PI3-k) and Akt were assessed by Western blot analysis. (a) Detection of four kinds of recombinant shPI3-k showed clearly the magnitude of shPik3c3-2 low expression compared to phosphate-buffered saline (PBS), negative shRNA and other PI3-k shRNA treatment, $**P < 0.01$ vs. PBS treatment group. (Similar results were also obtained, compared to other groups.) (b) Detection of four kinds of recombinant shAkt showed clearly the magnitude of shAkt 1-4 low-expression compared to PBS, negative shRNA and other Akt shRNA treatment. Pooling groups contained the four kinds of shRNA. These data were representative of three separate experiments: $**P < 0.01$ vs. PBS treatment group. (Similar results were obtained and compared to other groups.) (c) Rat GMCs were transfected with different doses of shRNAs for 54 h. Upper gel: Western blot by using two antibodies specific to T-Akt (tracks 1-4) and P*-Akt (tracks 5-7), respectively; lower gel: Western blot with an antibody to β -actin. The quantity of transfection in this experiment was 1/8, 1/4, 1/2 of original transfection quantity. Protein expression of T-Akt or P*-Akt normalized with β -actin in the GMCs transfected with shAkt1-4 (tracks 1-3) and shPik3c3-2 (tracks 5-7). Columns mean from three separate experiments.

transfected with shAkt1-4. Inhibition of GMC proliferation and fibronectin secretion induced by sublytic C5b-9 was carried out by knockdown of the corresponding target genes through the use of expression vectors of shPik3c3-2 or shAkt1-4 + shPik3c3-2.

Effects of shPik3c3-2 and shAkt1-4 on synthesis of Tsp-1 and TGF- β 1 induced by sublytic C5b-9

To examine the role of shPI3-k and shAkt on sublytic C5b-9-induced production of Tsp-1 and TGF- β 1, the GMCs were transfected with shPik3c3-2 or/and shAkt1-4, respectively, and then the cells were treated with sublytic

C5b-9 complexes. Expression of Tsp-1 or TGF- β 1 in GMCs transfected with shAkt1-4 + shPik3c3-2 + sublytic C5b-9 and shPik3c3-2 + sublytic C5b-9 in defined time was significantly inhibited compared with the sublytic C5b-9 group (Fig. 5), and in the shAkt1-4 + sublytic C5b-9 group, expression of Tsp-1 and TGF- β 1 were only slightly repressed. Tsp-1 and TGF- β 1 in GMCs treated with negative shRNA + sublytic C5b-9 were maintained at basal levels, which were equivalent to those of GMCs stimulated by sublytic C5b-9. These findings suggest that efficiency of shPik3c3-2 on repressing expression of Tsp-1 and TGF- β 1 induced by sublytic C5b-9 was better than that of shAkt1-4 alone.

Figure 4. Glomerular mesangial cell (GMC) proliferation and fibronectin (FN) production were inhibited by shRNA-mediated PI3-k and Akt gene silencing. mRNA and cyclin D2 protein (a, b) and FN (d, e) were assessed by real-time polymerase chain reaction and Western blot analysis. Expression of cyclin D2 and FN were relative to β -actin. Graphs are representative of three independent experiments. (c) Results of ^3H -thymidine incorporated into DNA for 18 h final incubation. These results are mean $\pm$ standard error from three independent experiments (* $P < 0.05$, ** $P < 0.01$ vs. sublytic C5b-9 treatment alone).

Discussion

Proliferation of mesangial cells is a feature of glomerular injury in several renal diseases, characterized by deposition of immunoglobulins and complement (4). Many studies suggest that excessive proliferation of GMCs is usually associated with ECM secretion and expansion, leading to

development of glomerulosclerosis (4,13,27). Rat anti-Thy 1 nephritis is an animal model for studying human mesangioproliferative glomerulonephritis, it is one of the immune complex-mediated renal diseases (3,7). In rat, it is induced by injection of anti-Thy 1 antibody against the Thy 1 antigen expressed in plasmamembranes of GMCs.

Figure 5. Knockdown of *phosphatidylinositol 3-kinase (PI3-k)* and *Akt* gene expression by short hairpin RNA (shRNA) reduced the level of Tsp-1 and TGF- β_1 production. Rat glomerular mesangial cells (GMC) were treated with negative shRNA, shAkt1-4, shPik3c3-2, shAkt1-4 + shPik3c3-2 or LY294002. (a) The GMCs were harvested and total RNA was isolated to measure Tsp-1 mRNA by real-time polymerase chain reaction (PCR). Three independent experiments at least were done for each result: * $P < 0.05$, ** $P < 0.01$ vs. sublytic C5b-9 treatment alone. (b) The GMCs were harvested and protein was isolated to perform Western blot by using antibodies against Tsp-1, total Akt (T-Akt), phosphorylated Akt (P*-Akt) and β -actin, respectively. Data are presented as mean $\pm$ standard error of three independent experiments (## $P < 0.01$, * $P < 0.05$, ** $P < 0.01$, $^xP < 0.05$, $^{xx}P < 0.01$ compared to the GMCs treated with sublytic C5b-9 alone). (c) Level of TGF- β_1 mRNA was measured by real-time PCR. (d) Content of activated TGF- β_1 in the supernatant of cultured GMCs was detected by ELISA kit. Data shown by (c) and (d) graphs are mean $\pm$ standard error (* $P < 0.05$, ** $P < 0.01$ vs. sublytic C5b-9 treatment alone).

Early studies demonstrated that inflammatory cell processes in the anti-Thy 1 nephritis model were complement-dependent and neutrophil independent (3,6,7). Injected anti-Thy 1 binds to its corresponding antigen, resulting in formation of immune complexes and activation of the complement system. Reaction of the complement cascade near GMC surface induces assembly of terminal components (namely, C5b-9). Complement C5b-9 complexes can form transmembrane channels or rearrangements of membrane lipid, with a loss of cell membrane integrity. However, injury by C5b-9 to nucleate cells is almost non-lysis (sublytic) as the surfaces of cells have many homologous restriction factors, such as Crry protein, CD59 and MCP (28–30). Complement activation is a pathogenic feature of human and animal immunological renal diseases. Subsequent experiments have confirmed that systemic complement depletion can reduce GMC proliferation and ECM secretion, as well as other pathological changes of renal tissue (10,31). Absence of C6 in the C⁻ PVG rat (complement C6 deficient) does not cause glomerular lesions after administration of anti-Thy 1 antibody as these rats are unable to make detectable C5b-9 complexes following complement reactions (6). Since several changes to GMCs can be induced by using anti-Thy 1 antibody (5%) and normal serum (4%) (9), in the present study, the GMCs-C5b-9 system was used as the model *in vitro* to observe the role of sublytic C5b-9 and relative signalling pathway activated by C5b-9 on GMC proliferation, ECM secretion, and synthesis of Tsp-1 and TGF- β_1 .

Sublytic C5b-9 is known to induce a variety of biological responses via the PI3-k/Akt pathway; for example, activation of PI3-k/Akt mediated by sublytic C5b-9 protects oligodendrocytes from death (32). PI3-k is a heterodimeric cytoplasmic enzyme that physically associates with tyrosine-phosphorylated membrane-bound cell proteins via its SH2 domain (85 KD regulatory subunit). Akt is one of the PI3-k effectors that play an important role in causing anti-apoptotic effects (33–35); Akt itself is a serine/threonine kinase that phosphorylates and regulates the activities of glycogen synthase kinase 3 and cyclin D2 (36–38). Previous studies lead to the hypothesis that translocation and membrane localization of PI3-k are necessary for its activation *in vivo*. Sublytic C5b-9 can activate heterotrimeric G proteins of Gi-Go subfamily (39) and transcription factors, including c-jun, jun-D and c-fos, with increasing AP-1 as well as JNK activity (32,40). Other studies have also reported that PI3-k/Akt has a positive role in cell proliferation induced by sublytic C5b-9 (34,35,41). Our previous experiments showed that the signalling pathway of PI3-k/Akt could be opened by sublytic C5b-9 stimulation, and expression of P*-Akt was decreased by using LY294002 to inhibit PI3-k activity (9).

Several approaches can be applied to specific inhibition of target gene expression at mRNA level, such as anti-sense, ribozyme, DNazymes and RNA interference. For RNA interference, viral vectors possess higher transduction efficiency than plasmids yet many investigators prefer to use plasmids as potential gene therapy agents as they can meet many of the prerequisites for clinically viable treatment: they are easy to manufacture, have low toxicity and are immunologically innocuous (thus allow repeated administration, and insensitivity to pre-existing immunity). Although the transfection efficiency of plasmids is currently still unsatisfactory, it may be improved or optimized by developing new delivery methods, such as new classes of cationic lipids and nanoparticles (42–44). Currently, there are some methods to produce shRNA, including chemical synthesis, *in vitro* transcriptional synthesis, and vector-expressing shRNA (45–48). In our experiment, the vector-expressing hairpin shRNA (PI3-k/Akt shRNA) was well constructed, PI3-k/Akt shRNA vectors could be stably transfected into the cultured GMCs and moreover, the efficiency and specificity of the shRNA plasmids that can knock down the corresponding target genes were confirmed to work very well. Since PI3-k/Akt is a mediator of survival (anti-apoptotic) signal, we chose *PI3-k/Akt* as target genes to observe whether PI3-k/Akt shRNA could inhibit GMC activation by sublytic C5b-9 stimulation. Our results revealed that PI3-k/Akt shRNA indeed reduced proliferation of GMCs and production of fibronectin, induced by sublytic C5b-9. Decrease of the parameters described above was more obvious in GMCs transfected with shPik3c3-2 or shPik3c3-2 + shAkt1-4 expression vectors.

GMCs attacked by sublytic C5b-9 can activate various metabolic processes, which induce multiple cytokines such as growth factors, basic fibroblast growth factor and platelet-derived growth factor (3). It has been reported that Tsp-1 affects nearly all phases of tissue repair and promotes cell proliferation (49,50). Moreover, excessive secretion of matrix protein from GMCs is always associated with overexpression of TGF- β_1 . TGF- β_1 is an important cytokine for induction of matrix synthesis in the kidney (51) and our previous studies confirmed that synthesis of Tsp-1 and TGF- β_1 in the GMCs stimulated by sublytic C5b-9, could be increased (9). In the present work, our data showed that production of Tsp-1 and TGF- β_1 induced by sublytic C5b-9 decreased when the GMCs were treated with PI3-k/Akt shRNA in advance. These results indicate that sublytic C5b-9 complexes are a potent stimulus for GMC activation and cytokine release, and the PI3-k/Akt signalling pathway may play an important role in the GMC reaction.

As far as the relationship between Tsp-1 or TGF- β_1 and cell proliferation or ECM secretion is concerned,

many pieces of research have demonstrated that Tsp-1 could induce cell proliferation and ECM secretion (11–13,16). In addition, Tsp-1 is an endogenous activator of TGF- β_1 (12,14). Our previous experiments found that when the GMCs were incubated with Tsp-1 blocking peptides (GGWSHW) + sublytic C5b-9 at the same time, production of activating TGF- β_1 was obviously decreased (9). However, inhibition of Tsp-1 or TGF- β_1 synthesis had no effect on GMC proliferation induced by sublytic C5b-9, but could markedly down-regulate expression of fibronectin (data not shown). Therefore, the molecular mechanisms of GMC proliferation and ECM secretion mediated by sublytic C5b-9 complexes need to be studied further in the future.

Conclusion

This study first explored the role of sublytic C5b-9 complexes on GMC proliferation and fibronectin production, and then demonstrated the effects of PI3-k/Akt signalling pathway on reactions including synthesis of Tsp-1 and TGF- β_1 in response to sublytic C5b-9 stimulation. The results indicate that sublytic C5b-9 not only can induce GMC proliferation and fibronectin secretion, but also can increase synthesis Tsp-1 and TGF- β_1 via the PI3-k/Akt signalling pathway. Taking these data into account, the findings imply that proliferative changes of GMCs in anti-Thy 1 nephritis are sublytic C5b-9 dependent, and the PI3-k/Akt pathway plays a role at least partially in the pathological processes.

Acknowledgements

We thank Professors Zhihong Qin and Qin Xue for reviewing the manuscript. We also thank Bin Sun for technical assistance. This study was supported by the National Natural Science Foundation of China (No. 30471615) and the Provincial Natural Science Foundation (No. 03KJA310074).

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