Retinoic acid-inducible gene-I is induced by double-stranded RNA and regulates the expression of CC chemokine ligand (CCL) 5 in human mesangial cells

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Abstract

Background. Retinoic acid-inducible gene-I (RIG-I) is a putative RNA helicase involved in immune reactions against RNA viruses and various inflammatory and auto-immune diseases. The purpose of the present study was to investigate the role of RIG-I in glomerular diseases.

Methods. We treated human mesangial cells in culture with polyinosinic–polycytidylic acid (poly IC), which is an authentic double-stranded RNA, and analysed the expression of RIG-I, CC chemokine ligand 5 (CCL5) and interferon (IFN)-β by western blotting, reverse transcriptase–polymerase chain reaction (RT–PCR) or enzyme-linked immunosorbent assay (ELISA). To elucidate the poly IC-signalling pathway, we subjected the cells to RNA interference (RNAi) against RIG-I, IFN-β or Toll-like receptor (TLR) 3. Furthermore, we studied the effects of IFN-β receptor blocking and IFN-β overexpression.

Results. Poly IC induced the expression of RIG-I and CCL5 in human mesangial cells, and RNAi against RIG-I inhibited this poly IC-induced CCL5 expression. Poly IC-induced RIG-I expression was also inhibited by RNAi against IFN-β and by an antibody against the IFN-β receptor. IFN-β overexpression induced the expression of both RIG-I and CCL5. The knockdown of TLR3 abolished poly IC-induced RIG-I expression.

Conclusions. The TLR3/IFN-β/RIG-I/CCL5 signalling pathway may mediate immune and inflammatory responses against viral infection in mesangial cells, suggesting the role of this pathway in the aggravation of glomerulonephritis due to viral infection.

Keywords: CC chemokine ligand 5; interferon-β; mesangial cells; polyinosinic–polycytidylic acid; retinoic acid-inducible gene-I

Introduction

Viral infection may sometimes trigger the development of an inflammatory renal disease or worsening of pre-existing renal disease [1]. Mesangial cells produce a wide variety of pro-inflammatory molecules that play an important role in immune and inflammatory reactions in the kidney; however, the details of the anti-viral reactions of mesangial cells are not yet fully elucidated.

Retinoic acid-inducible gene-I (RIG-I) encodes a DExH box protein, which is an RNA helicase [2]. The DExH box family proteins regulate RNA metabolism and have various biological functions [3]. In particular, RIG-I may detect viral RNAs and mediate immune reactions against RNA viruses [4]. RIG-I is also suggested to be involved in immune and inflammatory responses in various physiological and disease conditions [5].

A double-stranded RNA (dsRNA) is generated during replication cycle of certain viruses. Polyinosinic–polycytidylic acid (poly IC) is an authentic dsRNA, and both viral dsRNA and poly IC are potent inducers of type I interferons (IFNs) and the downstream molecules of the innate immune pathway. Therefore, poly IC has been widely used to mimic viral infection in various cell types, including dendritic cells [6], T-cells [7] and mesangial cells [8–11].
Poly IC stimulates mesangial cells to express functional molecules such as interleukin (IL)-6 [8], CC chemokine ligand (CCL) 2 (or monocyte-chemoattractant protein-1, MCP-1) [9], CCL5 (or regulated on activation, normal T-cell expression and secretion, RANTES) [10] and matrix metalloproteinase 9 (MMP9) [11].

We previously reported that poly IC induces the expression of RIG-I in human vascular endothelial cells [12] and human astrocytes [13]. We also found that IFN-γ induces the expression of RIG-I in human mesangial cells [14]. The present study was undertaken to evaluate the potential role of RIG-I in response to poly IC in human mesangial cells.

Materials and methods

Reagents
Poly IC and anti-actin rabbit IgG were from Sigma (St. Louis, MO, USA). The RNeasy total RNA isolation kit, small interfering RNAs (siRNA) against RIG-I (SI03019646) and Toll-like receptor (TLR) 3 (SI02655156), and non-silencing negative control siRNA (1027281) were from Qiagen (Hilden, Germany). Primer oligo(dT)12-18, dNTP mix, Moloney murine leukemia virus (MMLV) reverse transcriptase, Lipofectamine 2000 and siRNA against IFN-γ (5'-CCA GCU ACA ACU UGU UGG AUU-3' and 5'-AAU CCA AGG AAG UUG UAG CUC AUG G-3') were purchased from Invitrogen (Frederick, MD, USA). Taq DNA polymerase was from Promega (Madison, WI, USA). Oligonucleotide primers for polymerase chain reaction (PCR) were custom synthesized by Greiner Japan (Atsugi, Japan). Recombinant human (rh) IFN-β was from ProSpec (Rehovot, Israel). Anti-RIG-I antibody was raised as previously described methods [3]. Monoclonal antibody against type I IFN receptor was from CALBIOCHEM (La Jolla, CA, USA). FuGENE HD transfection reagent was from Roche Diagnostics (Basel, Switzerland). Dexamethasone was from Wako (Osaka, Japan). Enzyme-linked immunosorbent assay (ELISA) kits for CCL5 and IFN-β were from R&D Systems (Minneapolis, MN, USA) and Fujirebio (Tokyo, Japan), respectively.

Cells
Normal human mesangial cells and culture medium were purchased from Lonza (Walkersville, MD, USA), and the cells were cultured according to the manufacturer’s protocol [12]. Poly IC was dissolved in phosphate-buffered saline (PBS), and the cells were treated with 0.08 μg/mL of poly IC for up to 48 h. The cells were transfected with non-silencing control siRNA or siRNAs against RIG-I, IFN-β, or TLR3 by using Lipofectamine 2000 reagent. A pcDNA3.1-IFN-β expression construct was used for the transfection of normal human mesangial cells using FuGENE HD transfection reagent according to the manufacturer’s protocol.

RNA extraction and reverse transcription–PCR analysis
The total RNA was extracted from cells using RNeasy RNA extraction kit. Single-strand cDNA was synthesized from 1 μg of the total RNA by using oligo(dT)12-18 primer and MMLV reverse transcriptase. The cDNAs for RIG-I, CCL5, CCL2, IFN-α, IFN-β, IFN-γ, TLR3 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were amplified by PCR using Taq DNA polymerase. The PCR primers used are shown in Table 1.

Western blot analysis
Western blot analysis was performed as described in previous reports [3]. Briefly, the cells were lysed with Laemmli reducing sample buffer. The lysate was electrophoresed on a 7.5% or 10% polyacrylamide gel, and subsequently, the proteins were transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was incubated with an antibody against RIG-I (1:10 000 dilution) or actin (1:250 dilution), and subsequently with horseradish peroxidase-labelled anti-rabbit IgG. Immunodetection was performed using a chemiluminescence substrate.

ELISA for CCL5 and IFN-β
The concentration of CCL5 or IFN-β in the cell-conditioned medium was measured using ELISA kits.

Results

Poly IC induces the expression of RIG-I in cultured human mesangial cells
The treatment of mesangial cells with poly IC induced the expression of RIG-I in a concentration- and time-dependent manner (Figure 1). Stimulation with poly IC elevated the expression of both RIG-I mRNA and protein, reaching maximal expression levels ~16 and 24 h post-stimulation, respectively (Figure 1C and D). The expression of RIG-I protein remained high, but that of the mRNA decreased substantially 48 h after stimulation. Furthermore, the decay of RIG-I mRNA appeared to be faster than that of RIG-I protein.

Table 1. Oligonucleotide primers used in RT–PCR

<table>
<thead>
<tr>
<th>cDNA</th>
<th>Primers</th>
<th>Annealing temperature (°C)</th>
<th>Cycles</th>
<th>Product (bp)</th>
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</table>
| RIG-I | F: 5'-GCATATTGACTGACGGTGCA-3'   
R: 5'-CAGTCATGGGCTGACATTGTC-3' | 60 | 30 | 644 |
| CCL5 | F: 5'-CTACTCCGGAGGCTAAGGCAGAAGA  
R: 5'-GAGGGTTGTTAGAGGCGGGAAGC-3' | 58 | 30 | 318 |
| CCL2 | F: 5'-AAATCTGAGGCTCGACTCTCGC  
R: 5'-ATTTCGGGTGTTGAGTGAGT-3' | 58 | 30 | 353 |
| IFN-α | F: 5'-AGAATCTCTCTTCTCTCTCG-3'   
R: 5'-TCTGACAACCTCCCAGGCAC-3' | 55 | 34 | 350 |
| IFN-β | F: 5'-CCTTGAGGCAATTTGAAGGCAGC-3'  
R: 5'-CGAGCAGATGGAGCTTCTCCTT-3' | 57 | 34 | 370 |
| IFN-γ | F: 5'-AATGCAGGCTATTCAATGATG-3'   
R: 5'-TTGGACATCTCAATGCTGAT-3' | 55 | 34 | 270 |
| TLR3 | F: 5'-CTCGAAAGATACCCAGCAGG-3'   
R: 5'-CCATTATGAGCAATCTATGATG-3' | 60 | 35 | 287 |
| GAPDH | F: 5'-CCACCCCATGGCAATTCCATGGCA-3'  
R: 5'-TCTAGACGGCCAGTGTCACC-3' | 60 | 30 | 598 |
RIG-I siRNA suppresses poly IC-induced CCL5 expression in mesangial cells

The effect of RIG-I siRNA is shown in Figure 2. Treatment with RIG-I siRNA, which effectively inhibited RIG-I expression (Figure 2A), significantly lowered poly IC-induced CCL5 expression (Figure 2B and C). The concentration of CCL5 protein in the culture medium of poly IC-treated cells was 100 ± 12 pg/10⁶ cells, which was lowered to 35 ± 4 pg/10⁶ cells (mean ± SD, n = 3; P < 0.01, Student's t-test) after RIG-I knockdown (Figure 2C). The poly IC-induced expression of CCL2 mRNA was not affected by RIG-I siRNA (Figure 2B).

TLR3 is involved in poly IC-induced RIG-I expression

The poly IC-induced RIG-I expression was suppressed in response to treatment with siRNA against TLR3 (Figure 3A and B). Furthermore, TLR3 siRNA downregulated the poly IC-induced expressions of TLR3 and IFN-β, but RIG-I siRNA did not affect the expression of either TLR3 or IFN-β (Figure 3B). Treatment of mesangial cells with poly IC did not induce the expression of IFN-α or IFN-γ (Figure 3B).

Role of IFN-β in poly IC-induced RIG-I expression in mesangial cells

We next examined the role of IFN-β as a potential mediator of poly IC-induced RIG-I expression. The poly IC-induced expressions of IFN-β (Figure 4C) and RIG-I (Figure 4A and B) were markedly inhibited in cells transfected with IFN-β siRNA. Pre-treatment of the cells with a blocking antibody against type I IFN receptor also reduced the poly IC-induced expression of RIG-I (Figure 4D and E). Moreover, r(h)IFN-β induced the expression of RIG-I protein and mRNA (Figure 4F and G). The expression of both RIG-I and CCL5 was induced after transfection of the cells with IFN-β expression plasmid (Figure 5).
β is involved in the poly IC-induced RIG-I expression in human mesangial cells. The cells were transfected with siRNA against IFN-β or control siRNA, and after 24 h, they were treated with 20 μg/mL of poly IC. The cells were lysed, and the lysate was subjected to western blotting (A) and RT–PCR (B) after poly IC treatment for 24 and 16 h, respectively. (C) The culture medium was collected after the treatment of cells as in A and subjected to ELISA for IFN-β (n = 3, *P < 0.01). The cells were pre-treated with an anti-type I IFN receptor antibody (anti-IFN-R-Ab, 5 μg/mL) for 1 h and subsequently treated with poly IC (20 μg/mL). Western blotting (D) and RT–PCR (E) analyses for RIG-I were performed after poly IC treatment for 24 and 16 h, respectively. (F) The cells were treated with 2 ng/mL r(h)IFN-β for 24 h, and RIG-I protein was analysed by western blotting. (G) The cells were treated with r(h)IFN-β for 16 h, and RT–PCR analysis for RIG-I expression was performed.

Dexamethasone inhibits the induction of RIG-I by poly IC

Pre-treatment of cells with dexamethasone reduced the poly IC-induced expression of both RIG-I and IFN-β (Figure 6A and B), but this treatment had no effect on IFN-β-induced RIG-I expression (Figure 6C).

Discussion

It is believed that RIG-I controls immune and inflammatory responses by regulating the expressions of various downstream genes, including IFNs regulatory factor genes. It has also been suggested that RIG-I mediates the expression of CCL5 in poly IC-treated human astrocytes [13] and in rheumatoid synoviocytes treated with tumour-necrosis factor (TNF)-α [15]. However, in mesangial cells, RIG-I is not involved in poly IC-induced expression of IL-6 [8] or MMP9 [11]. In the present study, poly IC-induced CCL5 expression was partially inhibited in response to the knockdown of RIG-I. The expression of CCL2 was not affected by treatment with RIG-I siRNA, which may selectively regulate the expression of CCL5. CCL5 is a chemokine with chemotactic activity towards lymphocytes and monocytes; high levels of CCL5 mRNA are detected in urinary sediments of patients with lupus nephritis [16]. A recent report suggested that RIG-I, and not TLR3, mediates the secretion of type I IFN in poly IC-treated glomerular endothelial cells [17]. The cross-talk between glomerular endothelial cells and mesangial cells may be an important aspect of glomerular inflammation, and the RIG-I/CCL5 pathway in mesangial cells may contribute to glomerular inflammation, particularly after viral infection.

IFNs are cytokines with anti-viral properties and potent agonistic activity towards RIG-I expression [18]. Generally, type I IFNs, which include IFN-α and IFN-β, are involved in innate immune responses, while the type II IFN-γ is essential for adaptive immune responses. In our previous study, we found that IFN-γ enhances RIG-I expression in mesangial cells [14]. IFN-β is reported to mediate the induction of RIG-I in lipopolysaccharide-
stimulated RAW264.7 mouse macrophage-like cells [19] and in TNF-stimulated human synoviocytes [14]. Mesangial cells are known to produce IFN-β [8]. In the present study, treatment of mesangial cells with poly IC induced the expression of IFN-β but not the expression of IFN-α or IFN-γ. We found that siRNA-mediated knockdown of IFN-β was associated with the inhibition of poly IC-induced RIG-I expression. Blocking of TLR3 receptor with a specific antibody also inhibited the RIG-I expression. In addition, overexpression of IFN-β induced the expression of RIG-I and CCL5. These results suggest that newly synthesized IFN-β is involved in poly IC-induced RIG-I expression.

Both TLR3 and RIG-I are reported to serve as receptors for dsRNA [4,20]; furthermore, TLR3 is suggested to be involved in virus-associated renal inflammation [8,11]. In the present study, siRNA-mediated knockdown of TLR3 inhibited the poly IC-induced expression of both IFN-β and RIG-I. However, RIG-I knockdown had no effect on poly IC-induced IFN-β expression. Thus, RIG-I may function downstream to TLR3 in the signalling cascade activated by poly IC-induced expression of CCL5 in mesangial cells. In our previous study, we had observed high levels of RIG-I expression in the glomeruli and urinary sediments of patients with lupus nephritis [21,22]. RIG-I overexpression may be a pathological feature of renal inflammatory diseases, and CCL5 may serve as an effector molecule in such diseases.

Anti-inflammatory steroids are frequently used for the treatment of renal diseases, and dexamethasone was found to inhibit the poly IC-induced expression of IFN-β and RIG-I. Since dexamethasone had no effect on IFN-β-induced RIG-I expression, the inhibitory effect of dexamethasone may depend on the suppression of IFN-β production.

We conclude that poly IC induces the expression of RIG-I in cultured human mesangial cells, thereby upregulating CCL5 expression. TLR3 and newly synthesized IFN-β are involved in poly IC-induced RIG-I expression. On the basis of these results, we propose the TLR3/IFN-β/RIG-I/CCL5 pathway (Figure 7). The involvement of the TLR3/IFN-β/RIG-I/CCL5 pathway in poly IC signalling in mesangial cells is a novel finding, and this pathway may play an important role in immune and inflammatory reactions against viral infection in mesangial cells.

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Conflict of interest statement. None declared.

References

Treatment of db/db diabetic mice with triptolide: a novel therapy for diabetic nephropathy

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Abstract

Background. Current research on the progression of diabetic nephropathy (DN) suggests many important factors; metabolic disturbance, haemodynamic abnormality, chronic inflammation, oxidative stress, innate immune system activation and podocyte lesion. Triptolide, which is an active diterpene purified from the traditional Chinese medicine Tripterygium wilfordii Hook F (TwHF), has anti-inflammatory, anti-oxidative, immunosuppressive and podocyte-protective effects. Herein, we investigated the therapeutic effects of triptolide on DN in db/db diabetic mice and studied the potential mechanisms.

Methods. db/db mice with DN were administrated with triptolide or valsartan. After 4, 8 and 12 weeks of treatment, 24-h urine albumin level, blood biochemical parameters and body weight were measured. Glomerulus area, glomerulus volume to Bowman’s capsule volume ratio, podocyte changes and inflammatory and oxidative stress markers were quantitatively determined to evaluate renal lesions.

Results. The albuminuria in db/db diabetic mice was markedly attenuated after triptolide treatment, accompanied with alleviated glomerular hypertrophy and podocyte injury. In addition, the inflammation and oxidative stress in the kidneys were also attenuated, accompanied with improved hyperlipidaemia and obesity. The efficacy increased with the prolonging of triptolide treatment, and the efficacy in high-dose triptolide group was superior to that in the low-dose group. The effect of triptolide on glomerular hypertrophy was similar to valsartan, but the effects of triptolide on renal inflammation and oxidative stress were more profound than those of valsartan.

Conclusions. Triptolide can dramatically attenuate albuminuria and renal lesion accompanied with dyslipidaemia.

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