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Basic-helix-loop-helix transcription factor DEC2 constitutes negative feedback loop in IFN-β-mediated inflammatory responses in human mesangial cells

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ABSTRACT

Differentiated embryo-chondrocyte 2 (DEC2), a basic-helix-loop-helix transcriptional factor, is involved in various biological reactions by regulating the expression of its target genes. In the present study, we demonstrated DEC2 expression in response to the treatment with polyinosinic–polycytidylic acid (poly IC), an authentic double-stranded RNA, in cultured human mesangial cells. RNA interference against DEC2 enhanced the poly IC-induced expression of IFN-β and its downstream genes, retinoic acid-inducible gene-I (RIG-I) and CCL5. Knockdown of TLR3 abolished the poly IC-induced DEC2 expression. DEC2 expression may constitute a negative feedback system for the IFN-β/RIG-I/CCL5 pathway in the glomerulus, which may play a role in controlling protracted inflammatory reactions in the kidney.

1. Introduction

Differentiated embryo-chondrocyte 2 (DEC2; also named BHLHB3 or Sharp1), a member of the basic-helix-loop-helix transcriptional factors, regulates the expression of various genes [1]. DEC2 has been implicated in the differentiation of chondrocytes [2] and skeletal muscles [3], and in other various biological processes including the circadian rhythm in the suprachiasmatic nucleus [4], cellular responses against hypoxia [5] and apoptotic cell death [6]. Hypoxia induces the expression of DEC2 which, in turn, suppresses the expression of vascular endothelial growth factor (VEGF) in human oral cancer HSC-3 cells and mouse sarcoma 180 cells [5].

A recent study showed that DEC2 is involved in the differentiation of Th2 type cells promoting the production of Th2-type cytokines [7]. Also tumor-necrosis factor-α (TNF-α), a proinflammatory cytokine, was demonstrated to induce the expression of DEC2 in human breast cancer MCF-7 cells [6]. These reports suggest that DEC2 is also involved in immune and inflammatory reactions; however, there is no report on the role of DEC2 in innate immune responses against virus and other micropathogens.

Viral infection triggers the onset of inflammatory renal diseases or aggravates pre-existing renal diseases [8]. Mesangial cells express pattern recognition molecules such as toll-like receptor 3 (TLR3) [9] and retinoic acid-inducible gene-I (RIG-I) [10], which recognize viral RNA and mediate antiviral responses in the kidney. Polyinosinic–polycytidylic acid (poly IC) is an authentic double-stranded RNA (dsRNA). Both of viral dsRNA and poly IC are highly effective in inducing type I interferons (IFNs) and its downstream innate immune reactions. Therefore, poly IC has been used as a mimic for viral infection and poly IC treatment of mesangial cells has been shown to induce various inflammatory molecules [9,11]. We previously reported that poly IC activates the IFN-β/RIG-I/CCL5 pathway in human mesangial cells [10]. The present study was undertaken to address the involvement of DEC2 in immune and inflammatory responses in mesangial cells, particularly in the poly IC-induced activation of the IFN-β signaling.

2. Materials and methods

2.1. Reagents

Poly IC and anti-actin rabbit IgG were from Sigma (St. Louis, MA, USA). An RNeasy total RNA isolation kit, small interfering RNA

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Table 1
Oligonucleotide primers for RT-PCR.

cDNA	Primers	Annealing (°C)	Cycles	Product (bp)
DEC2	F: 5′-ATGCTCGACAGGCTTAGGACA-3′ R: 5′-TGTGTGAGCTGAGACATGAAAC-3′	55	30	612
IFN-β	F: 5′-CCTGTGGCAATTGAATGGGAGGC-3′ R: 5′-CCAGGCACAGTGACTGTACTCCTT-3′	57	33	370
TLR3	F: 5'-CTCAGAAGATTACCAGCCGCC-3′ R: 5'-CCATTATGAGACAGATCTAATG-3′	60	34	287
RIG-I	F: 5′-GCATATTGACTGGACGTGGCA-3′ R: 5′-CAGTCATGGCTGCAGTTCTGTC-3′	60	28	644
GAPDH	F: 5′-CCACCCATGGCAAATTCCATGGCA-3′ R: 5′-TCTAGACGGCAGGTCAGGTCCACC-3′	60	28	598

Table 2
Oligonucleotide primers for real-time quantitative RT-PCR.

cDNA	Primers	Annealing (°C)	Cycles	Product (bp)
DEC2	F: 5′-CGCCCATTCAGTCCGACTT-3′ R: 5′-CGGGAGAGGTATTGCAAGACTT-3′	55	40	78
IFN-β	F: 5′-CCTGTGGCAATTGAATGGGAGGC-3′ R: 5′-CCAGGCACAGTGACTGTACTCCTT-3′	57	40	370
RIG-I	F: 5′-GTGCAAAGCCTTGGCATGT-3′ R: 5′-TGGCTTGGGATGTGGTCTACTC-3′	55	40	115
CCL5	F: 5′-CTACTCGGGAGGCTAAGGCAGGAA-3′ R: 5'-GAGGGGTTGAGACGGCGGAAGC-3′	58	40	318
CCL2	F: 5′-AAACTGAAGCTCGCACTCTCGC-3′ R: 5′-ATTCTTGGGTTGTTGAGTGAGT-3′	58	40	353
18SrRNA	F: 5′-ACTCAACACGGGAAACCTCA-3′ R: 5′-AACCAGACAAATCGCTCCAC-3′	55	40	142

(siRNA) against TLR3 (SI02655156), RIG-I (SI03019646) or DEC2 (SI00312004) and non-silencing negative control siRNA (1027281) were from Qiagen (Hilden, Germany). Primer oligo(dT)$_{12-18}$, dNTP mix, MMLV reverse transcriptase, Lipofectamine RNAiMAX and siRNA against IFN-β (5′-CCA UGA GCU ACA ACU UGU UGG AUU-3′ and 5′-AAU CCA AGC AAG UUG UAG CUC AUG G-3′) were purchased from InVitrogen (Frederick, MD, USA). *Taq* DNA polymerase was from Promega (Madison, WI, USA). An iQSYBR Green kit was from Bio-Rad (Herculus, CA, USA). Oligonucleotide primers for polymerase chain reaction (PCR) were custom synthesized by Greiner Japan (Atsugi, Japan). Recombinant human (r(h)) IFN-β was from ProSpec (Rehovot, Israel). An Immobilon polyvinylidene difluoride membrane was from Millipore (Billerica, MA, USA). Rabbit polyclonal antibodies against DEC2 (H-72X) or TLR3 (H-125) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). An anti-RIG-I antibody was raised as described previously [12]. A SuperSignal west pico chemiluminescence substrate was from Thermo (Waltham, MA, USA). Enzyme-linked immunosorbent assay (ELISA) kits for IFN-β and CCL5 were from Fujirebio (Tokyo, Japan) and R&D Systems (Minneapolis, MN, USA), respectively.

2.2. Cells

Normal human mesangial cells and culture medium were purchased from Lonza (Walkersville, MD, USA), and the cells were cultured using MsBM^{TM} mesangial cell basal medium (Lonza) supplemented with 5% fetal bovine serum, 50 μg/ml gentamicin and 50 ng/ml amphotericin B [10]. The cells of the third to sixth passage were used for experiments. Poly IC was dissolved in PBS and the cells were treated with 0.08–50 μg/ml poly IC for up to 48 h. Transfection of the cells, at 30–50% confluence, with siRNA against TLR3, IFN-β, RIG-I or a non-silencing negative control was performed using a Lipofectamine RNAiMAX reagent. One day before the transfection, the medium containing antibiotics was replaced with the medium without antibiotics.

2.3. RNA extraction, reverse transcription-PCR (RT-PCR) and real-time PCR analyses

Total RNA was extracted from cells using an RNeasy RNA extraction kit. Single-strand cDNA was synthesized from 1 μg of total RNA using MMLV reverse transcriptase and oligo(dT)$_{12-18}$ primer. The cDNA for DEC2, IFN-β, TLR3, RIG-I, CCL5, CCL2, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or 18S rRNA was amplified using *Taq* DNA polymerase for RT-PCR or an iQSYBR Green kit for real-time PCR analysis. The primers used are shown in Tables 1 and 2.

2.4. Western blot analysis

Western blot analysis was performed as described [5,12]. Briefly, the cells were lysed with Laemmli's reducing sample buffer. The lysate was subjected to electrophoresis on a 10% or 7.5% polyacrylamide gel and proteins were transferred to an Immobilon polyvinylidene difluoride membrane. The membrane was incubated with an antibody against DEC2 (1:30,000 dilution), TLR3 (1:200 dilution), RIG-I (1:10,000 dilution) or actin (1:250 dilution), and subsequently with horseradish peroxidase-labeled anti-rabbit IgG. Immunodetection was performed using a SuperSignal west pico chemiluminescence substrate.

2.5. ELISA for IFN-β and CCL5

The concentration of IFN-β or CCL5 in cell-conditioned medium was measured using sandwich ELISA kits. Briefly, sample medium was pipetted into the wells with an immobilized capture antibody. The protein bound to the antibody was detected using the peroxidase-labeled secondary antibody and substrate. The optical density was determined using a microplate reader (Bio Rad iMark Microplate Reader, Bio Rad, Benica, CA, USA).

2.6. Statistical analyses

Statistical significance was analyzed by *t*-test.