COMMUNICATION

Double-stranded RNA induces galectin-9 in vascular endothelial cells: involvement of TLR3, PI3K, and IRF3 pathway

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Introduction

Galectins are a family of β-galactoside-binding animal lectins with conserved carbohydrate-binding domains (Hirabayashi and Kasai 1993), and galectin-9 has multiple immunomodulatory functions. Galectin-9 induces the chemotaxis of eosinophils (Matsumoto et al. 1998; Hirashima 1999, 2000), inhibits the function of Th1 cells via binding to T cell immunoglobulin (Ig)- and mucin-domain-containing molecule-3 (TIM-3) (Zhu et al. 2005), and promotes maturation of human monocyte-derived dendritic cells (Dai et al. 2006). Vascular endothelial cells play an important role in the initiation of inflammatory and immune reactions, and we found that the expression of galectin-9 in human umbilical vein endothelial cells (HUVECs) was stimulated by interferon (IFN)-γ (Imaizumi, Kumagai et al. 2002). The IFN-γ-induced galectin-9 expression was inhibited by 15-deoxy-Δ12,14-prostaglandin J3, which also exerts regulatory effects on inflammatory responses (Imaizumi et al. 2003).

Polyinosinic–polycytidylic acid (poly IC) is an authentic double-stranded RNA (dsRNA), and treatment of cells with poly IC mimics viral infection of the cells. In endothelial cells, poly IC induces the expression of various genes such as interleukin-6, vascular adhesion molecule-1, epithelial neutrophil activatin peptide-78, and retinoic acid-inducible gene-I (RIG-I) (Offerman et al. 1995; Imaizumi et al. 2005). We previously demonstrated that poly IC also induces the expression of galectin-9 in HUVECs (Ishikawa et al. 2004). However, receptors or signaling molecules involved in the poly IC-induced expression of galectin-9 have not been identified. Toll-like receptors (TLRs) recognize the molecular pattern of pathogens, which are important in innate immunity. TLR3 recognizes dsRNA, and binding of dsRNA to TLR3 activates the signaling to induce anti-viral responses (Alexopoulou et al. 2001). RIG-I, a putative RNA helicase (Imaizumi, Aratani et al. 2002), may also bind dsRNA and activate anti-viral responses independently of TLR3 (Yoneyama et al. 2005).

The present study was undertaken to examine whether TLR3 or RIG-I may be involved in the poly IC-induced expression of galectin-9 in HUVECs and whether other TLR ligands affect the galectin-9 expression. Poly IC is known to activate multiple signaling systems, including phosphatidylinositol 3-kinase (PI3K) and interferon regulatory factor 3 (IRF3) (Sarkar et al. 2004; Guillot et al. 2005; Yoneyama et al. 2005), and we also addressed the possible involvement of PI3K and IRF3 in poly IC-induced galectin-9 expression.

Results and discussion

dsRNA elicits various cellular responses similar to those provoked by viral infection. Vascular endothelial cells serve as one of the important components of immune and inflammatory reactions (McIntyre et al. 1997) and express various cytokines and adhesion molecules in response to dsRNA (Offerman et al. 1995; Imaizumi et al. 2005). Cellular recognition of dsRNA and subsequent activation of anti-viral signaling are important
for host defense against RNA viruses. TLR3 is localized on the endosome membrane and serves as a receptor for dsRNA, whereas RIG-I is a cytoplasmic molecule (Imaizumi et al. 2004) and is suggested to function as another receptor for dsRNA (Yoneyama et al. 2005). Both TLR3 and RIG-I independently turn on the anti-viral responses via activation of IRF3 (Yoneyama et al. 2005).

In the present study, we first examined the effect of short-interfering RNA (siRNA) against TLR3 or RIG-I on the poly IC-induced expression of galectin-9. In accordance with a previous report (Ishikawa et al. 2004), poly IC induced galectin-9 in HUVECs (Figure 1A). Poly IC also enhanced the expression of messenger ribonucleic acid (mRNA) for TLR3 and RIG-I. Transfection of the cells with siRNA against TLR3 or RIG-I markedly inhibited the expression of target mRNA, and the poly IC-induced galectin-9 expression was inhibited by knockdown of TLR3, but not of RIG-I (Figure 1A and B). Thus, TLR3 may be involved in the poly IC-induced galectin-9 expression in HUVECs. Expression of galectin-1 was not altered by poly IC treatment or transfection with the siRNAs.

Various signaling systems, including NF-κB, p38 MAPK, IRF3, and PI3K, are known to be involved in the gene expression induced by poly IC. We previously found that neither NF-κB nor p38 MAPK is involved in the endothelial expression of galectin-9 induced by poly IC (Ishikawa et al. 2004). In the present study, we examined whether IRF3 is involved in the galectin-9 induction by poly IC. Transfection of IRF3 siRNA markedly inhibited the IRF3 expression and poly IC-induced galectin-9 expression (Figure 1C and D). IRF3 may be involved in the galectin-9 expression mediated through TLR3. PI3K catalyzes the production of PI(3,4,5)P3, which is followed by phosphorylation of downstream kinases. A recent study showed that the PI3K signaling pathway is activated via TLR3 (Sarkar et al. 2004), and that LY294002, an inhibitor of PI3K, reduces the production of CC chemokine ligand 5 (CCL5) in response to poly IC (Guillot et al. 2005). In the present study, pretreatment of the cells with LY294002 inhibited the poly IC-induced galectin-9 expression (Figure 1E and F). Taken together, these results may imply that poly IC induces galectin-9 expression in HUVECs via TLR3, PI3K, and IRF3. Our results summarized in Figure 1G.

Next, we examined whether galectin-9 expression is regulated via other TLRs. We used Escherichia coli lipopolysaccharide (LPS), heat-killed Listeria monocytogenes (HKLM), loxoribine (LOX) or CpG oligonucleotide as ligands for TLR4, TLR2, TLR7, or TLR9, respectively. The effect of E. coli LPS on the expression of galectin-9 is summarized in Figure 2A–C. Treatment of HUVECs with LPS induced galectin-9 mRNA, and this was inhibited by knockdown of TLR4; however, LPS did not induce the expression of galectin-9.
protein. IFN-γ, used as a positive control, significantly induced the galectin-9 protein expression. This result suggests that certain post-transcriptional mechanisms may be necessary for the LPS-induced galectin-9 expression in HUVECs. HKLM and CpG oligonucleotide induced the expression of CCL5 mRNA, but not galectin-9 in HUVECs. LOX did not induce the galectin-9 expression in HUVECs, although it induced interleukin-8 (IL-8) mRNA. TLRs recognize the molecular pattern of the pathogens, and TLR3 recognizes dsRNA and may play an important role in the innate immunity against viral infection. TLR3 may be involved in galectin-9 expression; and this result suggests a certain role of galectin-9 in anti-viral responses in endothelial cells.

In conclusion, the TLR3, PI3K, and IRF3 pathway may be involved in poly IC-induced galectin-9 expression in HUVECs.

Materials and methods

Reagents

HUVECs were purchased from Cambrex (Walksville, MD). Humedia EB-2 and its supplements were from Kurabo (Osaka, Japan). Poly IC, LPS from E. coli, and anti-actin IgG were from Sigma (St Louis, MO). TLR agonists, LOX and CpG oligonucleotide M362, were from InvivoGen (San Diego, CA). Recombinant human [r(h)] IFN-γ was from Roche (Mannheim, Germany). LY294002, an inhibitor of PI3K, was from Cell Signaling Technology (Beverly, MA). Lipofectamine 2000, oligo(dT)12-18 and M-MuLV reverse transcriptase were from Invitrogen (Carlsbad, CA). SiRNAs (#SI012655156 for TLR3, #SI012657403 for RIG-I, #SI03117359 for IRF3, and #SI03095645 for TLR4), an RNase total RNA isolation kit and Taq DNA polymerase were purchased from Qiagen (Hilden, Germany). Oligonucleotide primers were from Fasmac (Kanagawa, Japan). Immobilon polyvinylidene difluoride (PVDF) membrane was from Millipore Japan (Tokyo, Japan). A chemiluminescent substrate Supersignal west femto was from Pierce (Rockford, IL).

Cell culture

HUVECs were cultured as described (Ishikawa et al. 2004). Briefly, HUVECs were cultured using HuMedia EB-2 supplemented with 2% fetal bovine serum, 10 ng/mL epidermal growth factor, 1 μg/mL hydrocortisone, 5 ng/mL basic fibroblast growth factor, and 10 μg/mL heparin. The cells of the first to the fifth passage were used for experiments. HUVECs were transfected with siRNA using Lipofectamine 2000 according to the manufacturer’s instructions. Briefly, the cells were maintained in the medium without antibiotics for 24 h, and then incubated with transfection mixture for 24 h. HUVECs were treated with poly IC, LPS, HKLM, LOX, CpG oligonucleotide M362, or r(h) IFN-γ. LY294002 was added to HUVECs 30 min before the application of poly IC.

Reverse transcription–polymerase chain reaction (RT–PCR)

RT–PCR analysis for the expression of galectin-9, galectin-1, TLR3, and RIG-I was performed as described previously (Imaizumi et al. 2002; Ishikawa et al. 2004). Total RNA was extracted from HUVECs using an RNeasy total RNA isolation kit. Single-stranded complementary deoxyribonucleic acid (cDNA) for a PCR template was synthesized from 1 μg total RNA using primer oligo(dT)12-18 and M-MuLV reverse transcriptase. Specific primers for galectin-9, galectin-1, TLR3, RIG-I, IRF3, TLR4, CCL5, IL-8, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were as follows:

galactin-9-F (5′-GAGATGGCCTTACGAGTTC-3′),
galactin-9-R (5′-CGCCTATCTGCTGACATGGT-3′),
galactin-1-F (5′-TGAGTCGCAGAATCTGCTA-3′),
galactin-1-R (5′-TAGTTTGGGCTCGGCATGGTGG-3′),
CCL5-F (5′-CTCAAGAGAATACGCGCC-3′),
CCL5-R (5′-CCTATTAGAAGAGATCTAATG-3′),
Ly-8-F (5′-CTACTCGGGAGCTAAAGGCAGGAA-3′),
Ly-8-R (5′-GGAGGGTGGACGGCGGAAGC-3′),
TLR3-F (5′-CATGTTGACTGAGCTGTCC-3′),
TLR3-R (5′-TCACCTGGTGGTCCATGCCC-3′),
RIG-I-F (5′-CACTGCTATCGTGAGCTGTCC-3′),
RIG-I-R (5′-CATGTTGACTGAGCTGTCC-3′),
IRF3-F (5′-TACGTGAGCAGCATGCTA-3′),
IRF3-R (5′-ATGGGAGGTGCTTGGAAAT-3′),
TLR4-F (5′-TGGACTGATCCAGGACCTGAC-3′),
TLR4-R (5′-GGAGGGTGGACGGCGGAAGC-3′),
CCL5-F (5′-CTACTCGGGAGCTAAAGGCAGGAA-3′),
CCL5-R (5′-GGAGGGTGGACGGCGGAAGC-3′),
IL-8-F (5′-AGAGTGCTAAAGAATGTCGA-3′),
IL-8-R (5′-GAATTCCTACCCTCTTCAA-3′),

Fig. 2. (A) HUVECs were treated with 10 μg/mL E. coli LPS for up to 24 h, and RT–PCR analysis for galectin-9 and GAPDH was performed. (B) siRNA against TLR4 or non-silencing control was transfected into HUVECs. After 24 h of transfection, the cells were treated with 10 μg/mL LPS for 16 h. Total RNA was extracted and RT–PCRs for galectin-9, TLR4, and GAPDH were performed as in (A). (C) HUVECs were treated with 10 μg/mL LPS or 10 ng/mL IFN-γ for up to 24 h. Western blot analysis for galectin-9 and actin was performed. (D) HUVECs were treated with HKLM (at a multiplicity of 10:1), 500 μM LOX, or 2.5 μM CpG oligonucleotide for 16 h, and expression of mRNA for galectin-9, CCL5, IL-8, and GAPDH was examined by RT–PCR analysis. Galectin-9 cDNA was used for a positive control of the reaction.
The reaction condition for galectin-9 was 1 × (94°C, 1 min), 26 × (94°C, 1 min; 62°C, 1 min; 72°C, 1 min), and 1 × (72°C, 10 min). The reaction condition for galactin-1, RIG-I, IRF3, IL-8, and GAPDH was similar to that for galectin-9 except that amplification was repeated for 30 cycles and the annealing temperature was 55°C. For the analysis of TLR3, TLR4, and CCL5, amplification was performed for 35 cycles with the annealing temperature at 58°C. The products were analyzed by electrophoresis on a 1.5% agarose gel containing ethidium bromide. There are three isoforms of galectin-9 depending on the length of the linker peptide, short, medium and long types; and the size of the amplified product for galectin-9 was 978 bp, which corresponds to the medium type. The size of the products for TLR3, galactin-1, RIG-I, IRF3, TLR4, CCL5, IL-8, and GAPDH was 287, 346, 644, 425, 449, 318, 219, and 696 bp, respectively.

Western blotting

Western blot analysis for galactin-9 and actin was performed as described previously (Imaizumi et al. 2002; Ishikawa et al. 2004). Briefly, cells were washed with 20 mM cold phosphate-buffered saline, pH 7.4, and lysed with Laemmli’s reducing sample buffer. The lysates were subjected to electrophoresis on a 4–20% gradient polyacrylamide gel, and the proteins were transferred to an Immobilon PVDF membrane. The membrane was incubated with rabbit anti-galectin-9 IgG (0.2 μg/mL) or anti-actin IgG, and immunodetection was performed using anti-rabbit IgG labeled with horseradish peroxidase and a Supersignal west femto chemiluminescent substrate.

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Conflict of interest statement

None declared.

Abbreviations

CCL5, CC chemokine ligand 5; cDNA, complementary deoxyribonucleic acid; dsRNA, double-stranded RNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HKLM, heat-killed Listeria monocytogenes; HUVECs, human umbilical vein endothelial cells; IFN, interferon; IL-8, interleukin-8; IRF3, interferon regulatory factor 3; LOX, lipoxygenase; LPS, lipopolysaccharide; mRNA, messenger ribonucleic acid; PI3K, phosphatidylinositol 3-kinase; poly IC, polyinosinic–polycytidylic acid; PVDF, polyvinylidene difluoride; rhIFN-γ, recombinant human interferon-γ; RIG-I, retinoic acid-inducible gene-I; RNAl, RNA interference; siRNA, short-interfering RNA; TLR, Toll-like receptor.

References


