Neuroscience Research xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Neuroscience Research

journal homepage: www.elsevier.com/locate/neures

ISG54 and ISG56 are induced by TLR3 signaling in U373MG human astrocytoma cells: Possible involvement in CXCL10 expression

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ARTICLE INFO

Article history:
Received 5 December 2013
Received in revised form 8 February 2014
Accepted 1 March 2014
Available online xxx

Keywords:
TLR3
IFN-β
ISG54
ISG56
CXCL10
U373MG cells

ABSTRACT

Toll-like receptor (TLR) 3 is a pattern recognition receptor that recognizes double-stranded RNA (dsRNA). TLR3 signaling in astrocytes leads to the expression of interferon-β (IFN-β), and IFN-β regulates immune and inflammatory reactions by inducing IFN-stimulated genes (ISGs). We demonstrated in the present study that polyinosinic-polycytidylic acid (poly IC), an authentic dsRNA, up-regulated the expression of ISG54 and ISG56 in U373MG human astrocytoma cells. This reaction was confirmed to be mediated via the TLR3/IFN-β pathway. We also found that ISG56 positively regulates the expression of ISG54, retinoic acid-inducible gene-I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5). In addition, positive feedback loops were found between ISG54 and ISG56, and also between ISG54 and RIG-I. RNA interference experiments revealed that all of ISG54, ISG56, RIG-I and MDA5 were involved in the poly IC-induced expression of a chemokine CXCL10. These results suggest that ISG54 and ISG56 are involved in the induction of CXCL10 in TLR3/IFN-β signaling at least partly by co-operating with RIG-I and MDA5. ISG54 and ISG56 may contribute to immune and inflammatory reactions elicited by the TLR3/IFN-β signaling pathway in astrocytes, and may play an important role both in antiviral immunity and in neuroinflammatory diseases.

1. Introduction

Toll-like receptors (TLRs) function as pattern recognition receptors that recognize pathogen-associated molecular patterns (PAMPs) derived from infected microorganisms, and are key molecules in the initial step of innate immune system. Upon activation, TLRs trigger a cascade of intracellular signaling pathway, leading to the induction of molecules that regulate immune and inflammatory reactions (Zeytun et al., 2010). Recently, it is thought that TLRs also recognize damage-associated molecular patterns (DAMPs) of endogenous molecules that are released from damaged tissues. TLRs are expressed in the central nervous system (Hanke and Kielian, 2011), and play important roles not only in physiological host defense but also in neurological disorders such as stroke and injuries (Lee et al., 2013). In addition, TLR signaling is associated with various neurological reactions including neurogenesis, axonal growth, neuronal plasticity and behaviors (Okun et al., 2011).

TLR3 is a member of TLRs, and recognizes double-stranded RNA (dsRNA). Binding of dsRNA to TLR3 is followed by the activation of intracellular signaling cascades and leads to the production of type I interferons (IFNs), which are key cytokines in antiviral immunity. Subsequently, IFNs exert antiviral effects by inducing hundreds of IFN-stimulated genes (ISGs).

ISG54 (also named as IFN-induced protein with tetratricopeptide repeats 2; IFIT2) and ISG56 (also named as IFIT1) are members of ISGs, and encode multifunctional proteins with tetratricopeptide repeats (Fensterl and Sen, 2011). ISG54 and ISG56 interact with various proteins and RNAs, and exert a wide variety of biological

Abbreviations: DAMPs, danger-associated molecular patterns; IFN, interferon; ISG, interferon-stimulated gene; MDA5, melanoma differentiation-associated gene 5; PAMPs, pathogen-associated molecular patterns; poly IC, polyinosinic-polycytidylic acid; PRRs, pattern recognition receptors; RIG-I, retinoic acid-inducible gene-I; TLR, Toll-like receptor.

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http://dx.doi.org/10.1016/j.neures.2014.03.001
0168-0102/

functions including regulation of translation, inhibition of viral replication, cell migration, cell proliferation and cell death (Fensterl and Sen, 2011; Reich, 2013).

Astrocytes play important roles in homeostasis, glutamate metabolism, energy production, inflammatory reactions and responses to injury in the central nervous system (Sofroniew and Vinters, 2010). TLR3 is expressed in astrocytes, and the activation of astroglial TLR3 induces the expression of various proinflammatory cytokines and chemokines including CXCL10 (Farina et al., 2005). TLR3 signaling in astrocytes induces the expression of IFN-β, a major type I IFN expressed in astrocytes (Rivieccio et al., 2006), and IFN-β elicits diverse immune and inflammatory reactions by inducing the expression of various effectors encoded by IFN-stimulated genes (ISGs) (Schittone et al., 2012).

To date, the roles of ISGs in astrocytes have not been well investigated. We have previously reported that U373MG astrocytoma cells express ISG56 in response to TLR4 signaling, and ISG56 positively regulates the expression of CXCL10 induced by *Escherichia coli* lipopolysaccharide (Imaizumi et al., 2013b). However, the role of ISG56 in TLR3 signaling is not known. In addition, there have been no reports on the expression and function of ISG54 in astrocytes.

Retinoic acid-inducible gene-I (RIG-I) (Imaizumi et al., 2002) and melanoma differentiation-associated gene 5 (MDA5) (Kang et al., 2002) are putative RNA helicases with DExH box (Schwer, 2001), and both are also members of ISGs. RIG-I and MDA5 can function as intracellular receptors for RNA virus (Yoneyama et al., 2005), and also as intracellular signaling molecules in the TLR3/IFN-β pathway in mesangial cells (Imaizumi et al., 2010, 2012). Thus, RIG-I and MDA5 are thought to be involved in antiviral immune and inflammatory reactions. We have previously reported that polyinosinic-polycytidylic acid (poly IC), a synthetic dsRNA, induces the expression of RIG-I (Yoshida et al., 2007), and that MDA5 constitutes a positive feedback loop with ISG56 in TLR4 signaling in U373MG cells (Imaizumi et al., 2013b). It is also reported that RIG-I is induced in murine glial cells exposed to vesicular stomatitis virus (Furr et al., 2008). However, relationship between ISG54/ISG56 and RIG-I/MDA5 in astrocytes are largely unknown.

This study was undertaken to examine the role of ISG54 and ISG56 in the downstream of TLR3 signaling in U373MG human astrocytoma cells used as a model of astrocytes, using poly IC as a TLR3 ligand. We also examined the role of ISG54 and ISG56 in the expression of CXCL10, and the relationship between ISG54/ISG56 and RIG-I/MDA5 in the TLR3/IFN-β pathway.

2. Materials and methods

2.1. Reagents

Dulbecco's modified Eagle medium (DMEM), M-MLV reverse transcriptase and Lipofectamine RNAiMAX reagent were purchased from Invitrogen (Frederick, MD, USA). Poly IC and an anti-actin rabbit IgG were from Sigma (St. Louis, MA, USA). Recombinant human IFN-β was from ProSpec (East Brunswick, NJ, USA). Small interfering RNAs (siRNAs) against TLR3 (SI02655156), ISG54 (SI04145372), ISG56 (SI02660777), RIG-I (SI03019646) or MDA5 (SI03649037), and non-silencing negative control siRNA (1027281) were from Qiagen (Hilden, Germany). siRNA against IFN-β was described previously (Imaizumi et al., 2010). A rabbit polyclonal antibody against MDA5 (29020) was from Immuno-Biological Laboratories (Takasaki, Japan). Rabbit antibodies against ISG54 (N1) or ISG56 (N2C3) were from GeneTex (Irvine, CA, USA). A rabbit polyclonal antibody against RIG-I was described previously (Imaizumi et al., 2002). The illustra RNAspin kit was from GE Healthcare (Buckinghamshire, England). dNTP mix was from Thermo Fisher Scientific (Asheville, MA, USA). SsoAdvanced SYBR Green Supermix was from Bio-Rad (Hercules, CA, USA). Oligonucleotide primers for polymerase chain reaction (PCR) were synthesized by Greiner Japan (Atsugi, Japan). An enzyme-linked immunosorbent assay (ELISA) kit for CXCL10 was from R&D systems (Minneapolis, MN, USA).

2.2. Cell culture

U373MG human astrocytoma cells (ECACC No. 89081403) were obtained from European Collection of Cell Cultures and cultured using DMEM supplemented with 10% fetal bovine serum as previously described (Yoshida et al., 2007). We have previously confirmed that U373MG cells have characteristics of astrocytes (Imaizumi et al., 2013b). The cells were treated with 10–50 μg/ml poly IC for up to 48 h. In RNA interference experiments, the cells were cultured with the medium without antibiotics for 24 h before the transfection. Then the cells were transfected with non-silencing control siRNA, siRNA against TLR3, IFN-β, RIG-I, MDA5, ISG54 or ISG56 using a Lipofectamine RNAiMAX reagent according to the manufacturer's protocols. After 48 h incubation, the cells were treated with 30 μg/ml poly IC for indicated period of time.

2.3. Western blot analysis

Western blot analysis was performed as described (Imaizumi et al., 2012). Briefly, the cells were lysed with Laemmli's reducing

Table 1
Oligonucleotide primers for real-time quantitative RT-PCR.

cDNA	Primers	Annealing (°C)	Cycles	Product (bp)
ISG54	F: 5′-GGTCTCTTCAGCATTTATTGGTG-3′ R: 5′-TGCCGTAGGCTGCTCTCCA-3′	60	40	144
ISG56	F: 5′-TAGCCAACATGTCCTCACAGAC-3′ R: 5′-TCTTCTACCACTGGTTTCATGC-3′	60	40	394
TLR3	F: 5′-TGTCTGGAAGAAAGGGACTTTGA-3′ R: 5′-GTTGAACTGCATGATGTACCTTGA-3′	60	40	154
IFN-β	F: 5′-CCTGTGGCAATTGAATGGGAGGC-3′ R: 5′-CCAGGCACAGTGACTGTACTCCTT-3′	60	40	370
RIG-I	F: 5′-GTGCAAAGCCTTGGCATGT-3′ R: 5′-TGGCTTGGGATGTGGTCTACTC-3′	55	40	115
MDA5	F: 5′-GTTGAAAAGGCTGGCTGAAAAC-3′, R: 5′-TCGATAACTCCTGAACCACTG-3′,	60	40	468
CXCL10	F: 5′-TTCAAGGAGTACCTCTCTCTAG-3′ R: 5′-CTGGATTCAGACATCTCTTCTC-3′	60	40	177
GAPDH	F: 5′-GCACCGTCAAGGCTGAGAAC-3′ R: 5′-ATGGTGGTGAAGACGCCAGT-3′	60	40	142

Please cite this article in press as: Imaizumi, T., et al., ISG54 and ISG56 are induced by TLR3 signaling in U373MG human astrocytoma cells: Possible involvement in CXCL10 expression. Neurosci. Res. (2014), http://dx.doi.org/10.1016/j.neures.2014.03.001