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Desferrioxamine, an iron chelator, inhibits CXCL10 expression induced by polyinosinic–polycytidylic acid in U373MG human astrocytoma cells

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ABSTRACT

Although iron is essential in physiological processes, accumulation of iron in central nervous system is associated with various neurological diseases including Alzheimer's disease and Parkinson's disease. Innate immune reactions are involved in the pathogenesis of those diseases, but roles of iron in innate immunity are not known well. In the present study, pretreatment of U373MG human astrocytoma cells with an iron chelator desferrioxamine (DFX) inhibited the expression of CXCL10 induced by a Toll-like receptor 3 (TLR3) agonist polyinosinic–polycytidylic acid (poly IC). Induction of interferon-β (IFN-β) was not affected, but phosphorylation of signal transducer and transcription 1 (STAT1) was decreased by DFX. We have previously reported that various IFN-stimulated genes (ISGs) are involved in CXCL10 induction by poly IC. Pretreatment with DFX also decreased the expression of these ISGs. Pretreatment of cells with $FeSO_4$ counteracted inhibitory effects of DFX on ISG56, retinoic acid-inducible gene-I (RIG-I), CXCL10 and phosphorylation of STAT1. These results suggest that iron may positively regulate STAT1 phosphorylation and following signaling to express ISG56, RIG-I and CXCL10 in U373MG cells treated with poly IC. Iron may contribute to innate immune and inflammatory reactions elicited by the TLR3 signaling in astrocytes, and may play an important role in neuroinflammatory diseases.

1. Introduction

Iron is essential in many physiological processes, including DNA replication, oxygen transport, mitochondrial electron transport, myelination, glucose metabolism and neurotransmitter synthesis (Crichton et al., 2011). However, increased levels of iron in specific region of the central nervous system may generate oxidative stress and oxidation of cellular components and neuronal cell death (Andersen et al., 2014). Dysregulation of iron homeostasis and enhanced accumulation of iron have been found in the brains of patients with Alzheimer's disease (Raven et al., 2013), Parkinson's disease (Weinreb et al., 2013), multiple sclerosis (Stankiewicz et al., 2014) and many other neurodegenerative diseases.

Innate immune reactions and subsequent inflammation are important in host defense against infection of microbes in the central nervous system. Moreover, inflammatory reactions are known to be involved in various neurological diseases. The initial step of innate immune reactions is recognition of exogenous pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors. In addition to PAMPs, cellular components released from damaged or dying cells, designated as damage-associated molecular patterns (DAMPs), can be endogenous agonists for pattern recognition receptors (Kigerl and Popvich, 2009).

Abbreviations: DFX, desferrioxamine; IFN, interferon; ISG, IFN-stimulated gene; MDA5, melanoma differentiation-associated gene 5; RIG-I, retinoic acid-inducible gene-I; poly IC, polyinosinic–polycytidylic acid; TLR3, Toll-like receptor 3.

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Toll-like receptor 3 (TLR3) is a member of pattern recognition receptors, and recognizes double-stranded RNA (dsRNA) derived from viruses. Binding of dsRNA to TLR3 leads to the production of type I interferons (IFNs), which are key cytokines in innate immunity. Subsequently, IFNs induce hundreds of IFN-stimulated genes (ISGs) via activation of signal transducer and activator of transcription 1 (STAT1).

CXCL10 is a member of CXC chemokine family. Because the expression of CXCL10 can be induced by IFNs, CXCL10 is also named as IFN-inducible protein-10 (IP-10) and is designated as one of the ISGs. CXCL10 exerts its activity by binding its specific receptor CXCR3. In the central nervous system, CXCL10 mediates the infiltration of CXCR3-positive lymphocytes and activation of CXCR3-positive microglia, and CXCL10 contributes to diverse pathologic conditions (Muller et al., 2010).

Astrocytes have a variety of functions including formation of blood–brain barrier, transportation of nutrients and maintenance of ion balance. In addition, astrocytes play an important role not only in physiological host defense but also in various diseases related with neuro-inflammation (Sofroniew and Vinters, 2010). Pattern recognition receptors including TLR3 are expressed in astrocytes, and the activation of TLR3 in astrocytes results in the expression of proinflammatory chemokines including CXCL10 (Farina et al., 2007). Thus, expression of CXCL10 in astrocytes induced by TLR3 signaling may be important in anti-viral defense and neurological disorders related with inflammation.

Although iron is known to be involved in immune and inflammatory reactions, the role of iron in CXCL10 expression induced by TLR3 signaling in astrocytes is still unknown. Desferrioxamine (DFX) is an iron chelator, and is not only a useful tool to inhibit the function of iron in cells but also a neuroprotective reagent in animal models of Parkinson's disease (Zhang et al., 2012). The aim of the present study is to investigate the effect of DFX on CXCL10 expression induced by polyinosinic–polycytidylic acid (poly IC), an authentic ligand against TLR3, in U373MG astrocytoma cells.

We have previously reported that ISGs such as ISG56 (also named as IFN-induced protein with tetratricopeptide repeats 1; IFIT1), ISG54 (also named as IFIT2), retinoic acid-inducible gene-I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) are involved in CXCL10 expression induced by poly IC in U373MG astrocytoma cells (Imaizumi et al., 2014). Therefore, effect of DFX on the expression of these ISGs was also examined.

2. Materials and methods

2.1. Reagents

Dulbecco's modified Eagle medium (DMEM), Moloney murine leukemia virus (MMLV) reverse transcriptase and Lipofectamine RNAiMAX reagent were purchased from Invitrogen (Frederick, MD, USA). DFX, poly IC and an anti-actin rabbit IgG were from Sigma (St. Louis, MA, USA). Ferrous sulfate ($FeSO_4$) was from Wako (Osaka, Japan). Small interfering RNA (siRNA) against hypoxia-inducible factor-1α (HIF-1α) (SI02664053) and non-silencing negative control siRNA (1027281) were from Qiagen (Hilden, Germany). The illustra RNAspin kit was from GE Healthcare (Buckinghamshire, UK). 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). Rabbit antibodies against STAT1 (9172) or phosphorylated STAT1 (pSTAT1, 9171) were from Cell Signaling Technology (Beverly, MA, USA). 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). A rabbit polyclonal antibody against MDA5 (29020) was from Immuno-Biological Laboratories (Takasaki, 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., 2013). The cells were pretreated with 1–100 μM DFX for 1 h or 24 h, and subsequently treated with 30 μg/ml poly IC for 16 h. Treatment of cells with DFX and/or poly IC at these concentrations did not affect the viability of the cells (not shown). In a previous study, it was confirmed that poly IC effectively activated TLR3 signaling followed by induction of IFN-β, ISGs and CXCL10 at this concentration (Imaizumi et al., 2014). In a previous study (Imaizumi et al., 2014) and pilot studies, we have also confirmed that the expression levels of mRNA for IFN-β and ISG54 was maximal around 4 h after treatment of cells with poly IC, and those for ISG56, MDA5, RIG-I and CXCL10 were maximal around 8–16 h after poly IC treatment (not shown).

In RNA interference experiments, the cells were cultured with the medium without antibiotics for 24 h before the transfection. The cells were transfected with non-silencing control siRNA or siRNA against HIF-1α using a Lipofectamine RNAiMAX reagent according to the manufacturer's protocol, and were incubated for 48 h. The cells were treated with 100 μM DFX for 1 h, and subsequently with 30 μg/ml poly IC for additional 16 h.

In the experiments using $FeSO_4$, we pretreated the cells with 200 μM $FeSO_4$ for 24 h before treating cells with 100 μM DFX followed by 30 μg/ml poly IC. It was confirmed that treatment of cells with $FeSO_4$, DFX and poly IC did not affect the viability of cells in the condition tested (data not shown).

2.3. Quantitative real-time reverse transcription (RT)-PCR analysis

Total RNA was extracted from cells after the incubation, and single-strand cDNA as a PCR template was synthesized using oligo$(dT)_{12–18}$ primer and MMLV reverse transcriptase. The expression of mRNAs for vascular endothelial growth factor (VEGF), CXCL10, IFN-β, ISG56, ISG54, RIG-I, MDA5 and GAPDH were examined with a real-time RT-PCR system using SsoAdvanced SYBR Green Supermix. The primers used are shown in Table 1.

2.4. Western blot analysis

Western blot analysis was performed as described (Imaizumi et al., 2002). Briefly, the cells were lysed with Laemmli's reducing sample buffer, and the lysate was subjected to electrophoresis on a 7.5% or 10% polyacrylamide gel. The proteins were transferred to a polyvinylidene difluoride membrane, and the membrane was incubated with an antibody against pSTAT1 (1:1000), STAT1 (1:1000), ISG56 (1:5000), ISG54 (1:2500), RIG-I (1:10000), MDA5 (1:1000) or actin (1:5000), and subsequently with a horseradish peroxidase-labeled secondary antibody. Immunodetection was performed using a chemiluminescence substrate.

2.5. ELISA for CXCL10

Cell-conditioned medium was collected and centrifuged briefly, and the concentration of CXCL10 in the supernatant was determined using an ELISA kit according to the manufacturer's protocol.