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ORIGINAL ARTICLE

DESFERRIOXAMINE, AN IRON CHELATOR, INDUCES CXCL8 EXPRESSION IN U373MG HUMAN ASTROCYTOMA CELLS

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Abstract Dysregulation of iron homeostasis in brain causes various neurodegenerative disorders. In fact, high concentration of iron is present in brains of patients with Alzheimer's disease. It was previously reported that CXCL8 protects human neurons from amyloid-β-induced neurotoxicity and that astrocytes have the potential to play important roles in Alzheimer's disease. In the present study, we examined the effect of desferrioxamine, an iron chelator, on the expression of CXCL8 in U373MG human astrocytoma cells used as a model of astrocytes. Treatment of the cells with desferrioxamine induced the expression of CXCL8. Pretreatment of the cells with $FeSO_4$ counteracted the positive effect of desferrioxamine on CXCL8 production, suggesting that the effect of desferrioxamine was due to iron chelation. RNA interference experiments showed that HIF-1α was not involved in desferrioxamine-induced CXCL8 expression. We conclude that desferrioxamine induces CXCL8 in astrocytes and the chelation of iron may be a new therapeutic strategy for Alzheimer's disease.

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Key words: desferrioxamine; CXCL8; U373MG cells; iron; VEGF.

Introduction

Iron is essential in many important biological processes, such as oxygen transportation, DNA synthesis, mitochondrial respiration, myelin synthesis and neurotransmitter synthesis[1]. Therefore, dysregulation of iron homeostasis leads to various kinds of diseases. The deficiency of iron in the organism results in the development of several types anemia. On the other hand, the overload of iron can cause hemochromatosis, in which iron deposition at several organs drives to cirrhosis, hypogonadism, cardiomyopathy, arthropathy, diabetes mellitus and hepatocellular carcinomas[2]. Excessive accumulation of iron in tissues could result in the generation of reactive oxygen species, and leading to the induction of oxidative damage to cells and cell death[3]. Iron is of particular importance in brain functions, as well. Many neurodegenerative disorders, such as Alzheimer's disease, Parkinson's disease and multiple sclerosis, are also the consequence of dysregulation of brain iron homeostasis[1]. It has been reported that high concentrations of zinc, copper and iron are present in the insoluble amyloid plaques and neurofibrillary tangles in brain from patients with Alzheimer's disease[4]. Therefore, chelation of iron reduces the production of reactive oxidative species, and it may have protective effect on neuronal cell death. In addition, iron is involved in the expression of various genes and treatment of cells with iron chelator alters the expression of lots of molecules.

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CXCL8 is a chemokine which is known to induce chemotaxis of neutrophils, and plays a vital role in infectious diseases in central nervous system.

In addition to its chemotactic activities, CXCL8 is involved in multiple physiological and pathological reactions. CXCL8 plays a role in neuronal electrical activity, neurotransmitter release, synaptic plasticity, and also in the pathogenesis of multiple sclerosis and ischemic brain injury[5]. It was previously reported that CXCL8 protected human neurons from amyloid-β-induced neurotoxicity by increasing neuronal brain-derived neurotrophic factor[6]. Therefore, there is a possibility that CXCL8 and its receptors play a protective role in the pathogenesis of Alzheimer's disease.

Astrocytes are specialized glial cells that outnumber neurons by over five-fold. Reactive astrogliosis has the potential to play either primary or contributing roles in central nervous system disorders[7]. It is well known that inflammatory cytokines such as tumor-necrosis factor-α and interleukin-β induce the expression of CXCL8 in cultured human astrocytes[8]. In a previous study, we found that an iron chelator desferrioxamine, also named as deferoxamine, inhibited the expression of CXCL10 induced by Toll-like receptor 3 activation in U373MG astrocytoma cells[9]. However, the effect of iron chelation on CXCL8 expression in astrocytes is not known. This study was undertaken to examine the effect of desferrioxamine on the expression of CXCL8 in U373MG cells.

Materials and methods

Reagents

Dulbecco's modified Eagle medium (DMEM), Lipofectamine RNAi MAX, dNTP and M-MLV reverse transcriptase were obtained from Invitrogen (Frederick, MD, USA). Desferrioxamine was from Sigma (St Louis, MA, USA). Ferrous sulfate ($FeSO_4$) was from Wako (Osaka, Japan). Short interfering RNA (siRNA) against hypoxia-inducible factor-1α (HIF-1α) and control nonsilensing siRNA were from Qiagen (Hilden, Germany). SsoFast EvaGreen Supermix were provided by Bio-Rad (Herculus, CA, USA). Oligonucleotide primers for polymerase chain reaction (PCR) were purchased from Greiner Japan (Atsugi, Japan). An enzyme-linked immunosorbent assay (ELISA) kit for CXCL8 was obtained from R&D systems (Minneapolis, MN, USA).

Cell culture

U373MG human astrocytoma cells (ECACC No. 89081403) were purchased from European Collection of Cell Cultures and cultured using DMEM supplemented with 10% fetal bovine serum as previously described[10]. It was previously confirmed that U373MG cells have characteristics of astrocytes[11].

In order to examine the effect of desferrioxamine, we treated U373MG cells with up to 100 μM desferrioxamine and cultured for up to 45 h. In the experiments using $FeSO_4$, the cells were pretreated with 200 μM $FeSO_4$ for 24 h before being treated with 100 μM desferroxamine. In RNA interference experiments, the cells were transfected with siRNA against HIF-1α or control siRNA according to the manufacturer's protocol. After 48 h incubation, the cells were treated with 100 μM desferrioxamine.

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

Total RNA was extracted from the cells after the incubation. Subsequently, synthesis of single-strand cDNA was performed using M-MLV reverse transcriptase. The cDNA was then used as a PCR template. The expression levels of CXCL8 mRNA, vascular endothelial growth factor (VEGF) mRNA and 18S rRNA were determined with a real-time RT-

Table 1 Primers used for real-time PCR analyses

cDNA	sequences
CXCL8	F: 5'-AGGAGTGCTAAAGAACTTCGA -3',
	R: 5'-TGAATTCTCAGCCCTCTTCAA-3',
VEGF	F: 5′ -TGGAGTGTGTGCCCACTGAG-3′
	R: 5′ - TGCATTCACATTTGTTGTGCTGTAG-3′
18S rRNA	F: 5′ -ACTCAACACGGGAAACCTCA-3′
	R: 5′ -AACCAGACAAATCGCTCCAC-3′

PCR system using SsoAdvanced SYBR Green Supermix. The primers used in the present study are shown in Table 1.

ELISA for CXCL8

After the incubation, cell-conditioned medium was collected and centrifuged. We quantified the concentration of CXCL8 in the supernatant with an ELISA kit according to the manufacturer's protocol.

Statistics

The results of real-time RT-PCR and ELISA are expressed as means +/- SD. Data was analyzed by *t*-test, and differences were considered significant at $p < 0.05$.

Results

Desferrioxamine induces the expression of CXCL8 in U373MG astrocytoma cells

U373MG cells were treated with 50 or 100 μM desferrioxamine, incubated for 20 h, and the expression of CXCL8 was evaluated. Expression of VEGF was examined as a positive control. A certain amount of CXCL8 mRNA and protein was expressed in U373MG cells under resting condition. Treatment of the cells with desferrioxamine significantly increased the expression of CXCL8 mRNA, as well as that of VEGF mRNA, in a concentration-dependent manner (Fig. 1A). The level of CXCL8 protein in the conditioned medium was increased by treatment of cells with 100 μM desferrioxamine (Fig. 1B).

CXCL8 mRNA began to increase 4 h after treatment with desferrioxamine, reached maximal level at 24 h and decreased thereafter (Fig. 1C). On the other hand, the concentration of CXCL8 protein in the medium from desferrioxamine-treated cells was significantly higher than that from untreated cells at 24 or 45 h (Fig. 1D).

$FeSO_4$ counteracted the effect of desferrioxamine on the expression of CXCL8

Next, we examined the effect of $FeSO_4$ on the effect of desferrioxamine.

None of the expression levels for CXCL8 mRNA, CXCL8 protein or VEGF mRNA was affected by treatment of U373MG cells with 200 μM $FeSO_4$ alone (Fig. 2). However, pretreatment of cells with $FeSO_4$ for 24 h significantly counteracted the desferrioxamine-induced increase of CXCL8 mRNA, CXCL8 protein and VEGF mRNA (Fig. 2).

HIF-1α is not involved in the upregulation of CXCL8 induced by desferrioxamine

Transfection of cells with siRNA against HIF-1α significantly inhibited the expression of HIF-1α mRNA (Fig. 3, upper panel). Although knockdown of HIF-1α resulted in partial inhibition of VEGF expression induced by desferrioxamine (Fig. 3, lower panel), induction of CXCL8 mRNA was not affected (Fig. 3, middle panel).

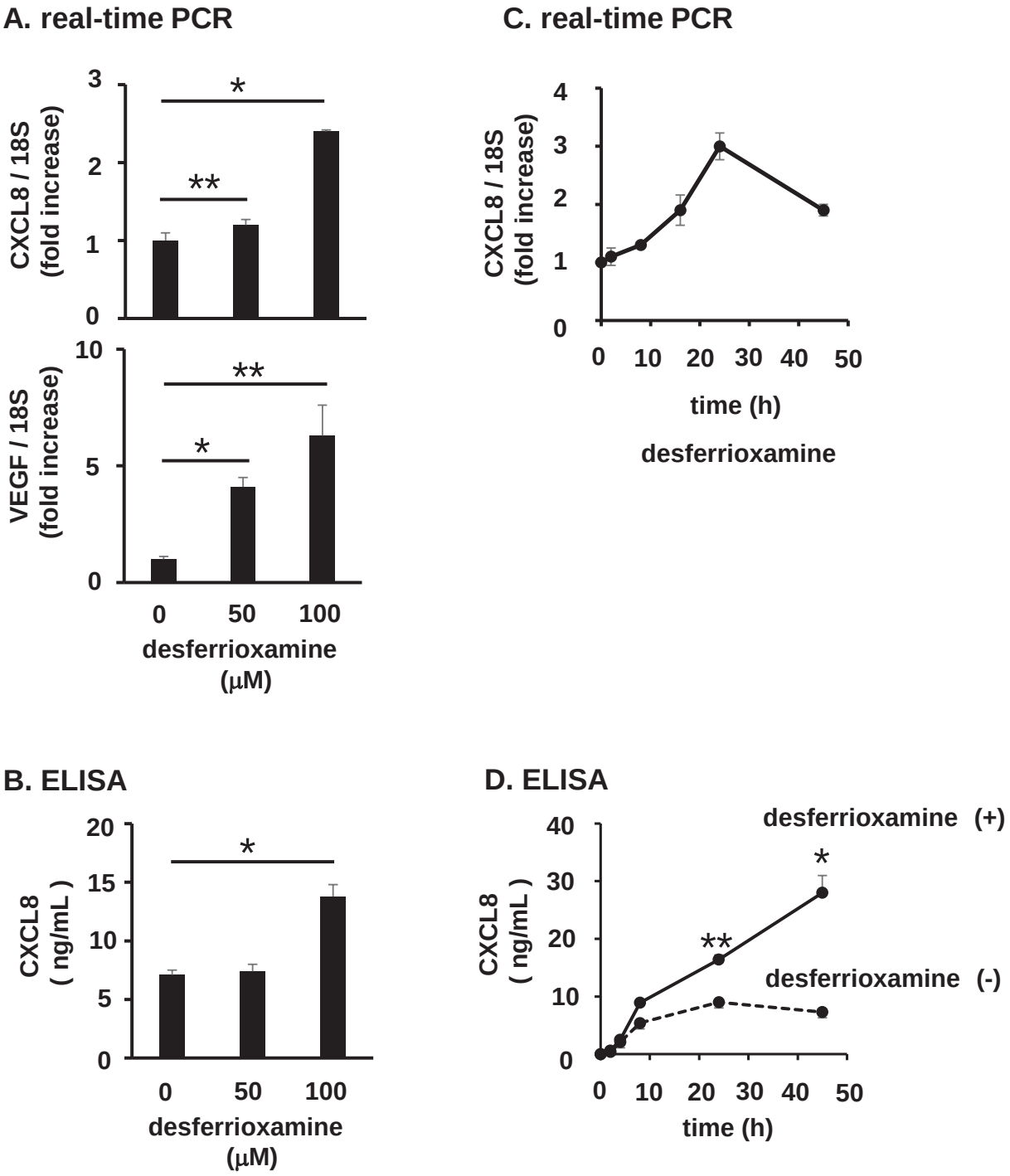

Fig.1 Treatment of U373MG human astrocytoma cells with desferrioxamine induced CXCL8 and VEGF in concentration- and time-dependent manners.

(A) The cells were treated with 50 or 100 μM desferrioxamine and cultured for 20 h. Total RNA was extracted from the cells after the incubation, and cDNA was synthesized from the total RNA. The cDNA for CXCL8, VEGF or 18S was amplified and analysed by quantitative real-time PCR (*$p < 0.01$, **$p < 0.05$, n=3). (B) The cells were treated with desferrioxamine as in (A), and the cell-conditioned medium was collected. The concentration of CXCL8 in the medium was measured using an ELISA kit (*$p < 0.01$, n=3). (C) The cells were treated with 100 μM desferrioxamine and cultured for up to 45 h. The expression of mRNA for CXCL8 was examined by quantitative real-time PCR analysis. (D) The cells were treated and cultured as in (C), and the level of CXCL8 protein in the conditioned medium was measured by an ELISA (*$p < 0.01$, **$p < 0.05$, compared with the cells without desferrioxamine treatment, n=3).

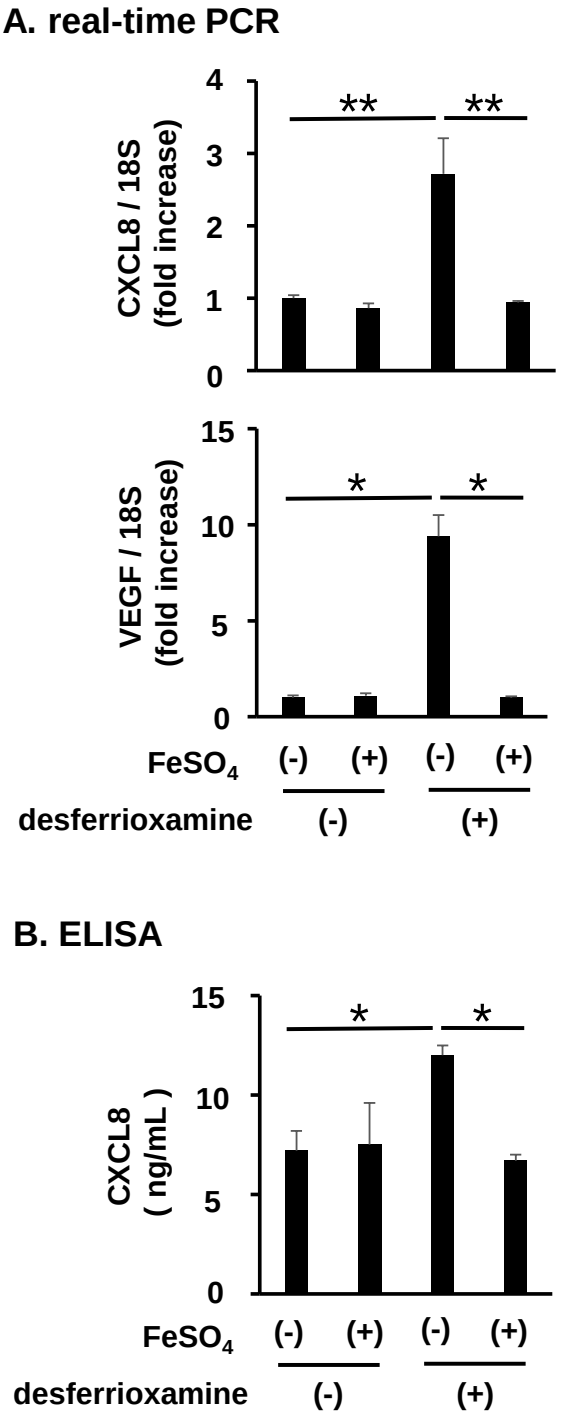

Fig.2 $FeSO_4$ counteracted the inducible effect of desferrioxamine on the expression of CXCL8 and VEGF.

The cells were pretreated with 200 μM $FeSO_4$ for 24 h before being treated with 100 μM desferrioxamine for an additional 20 h. Real-time RT-PCR analysis (A) and ELISA (B) were performed as in Fig. 1 ($^{*}p < 0.01$, $^{**}p < 0.05$, n=3).

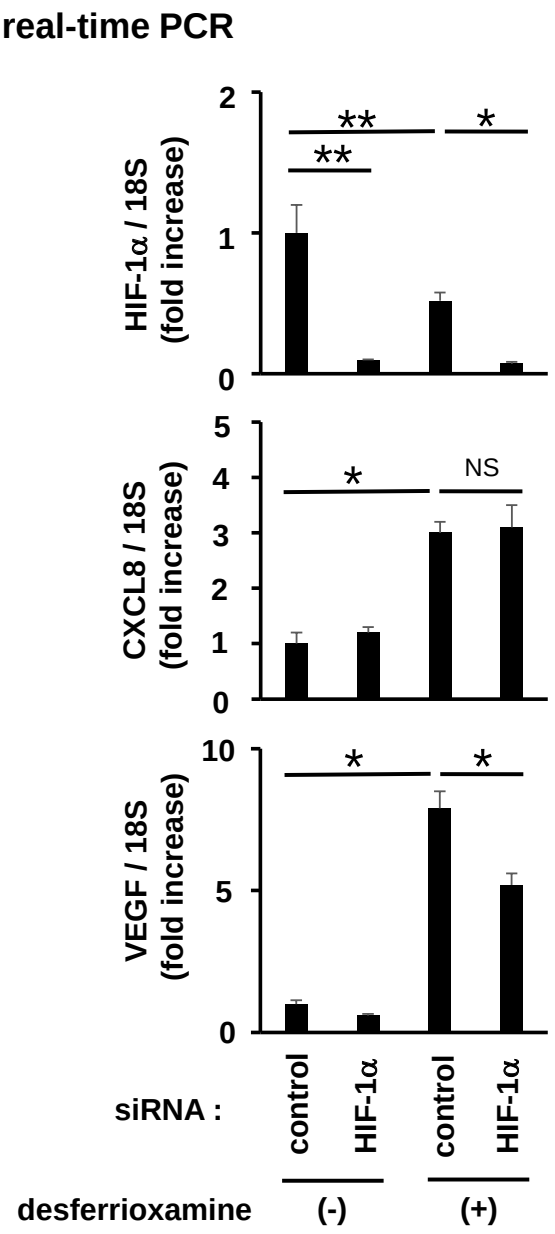

Fig.3 HIF-1α is not involved in the induction of CXCL8 induced by desferrioxamine.

The cells were transfected with siRNA against HIF-1α or control siRNA. After 48 h incubation, the cells were treated with 100 μM desferrioxamine for an additional 20 h. Then the cells were subjected to real-time RT-PCR analysis as in Fig. 1 ($^{*}p < 0.01$, $^{**}p < 0.05$, NS; not significant, n=3).

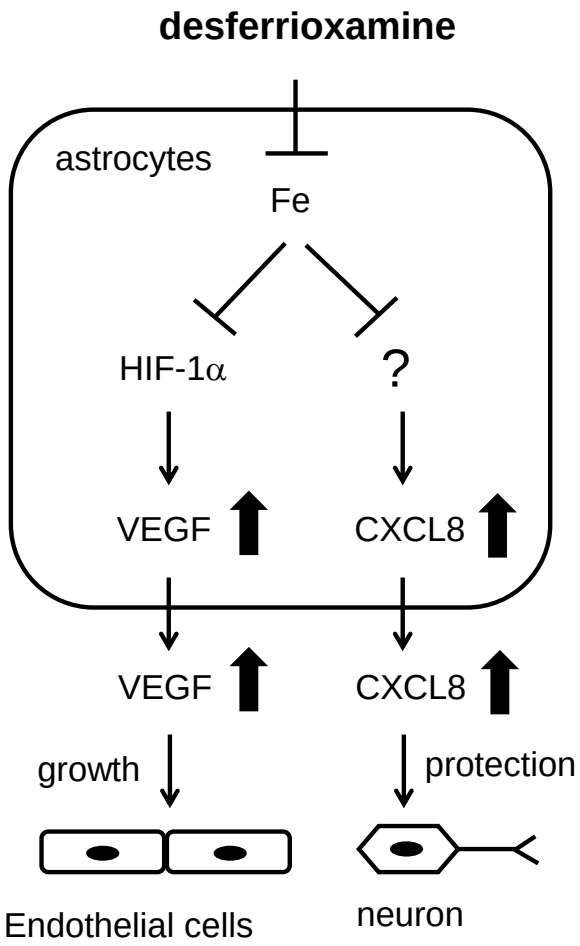

Fig.4 Supposed model of CXCL8 expression induced by desferrioxamine in astrocytes.
When astrocytes are treated with desferrioxamine, iron (Fe) is chelated. Iron chelation leads to VEGF expression via activation of HIF-1α, and VEGF secreted from astrocytes may induce endothelial growth. On the other hand, iron-chelation results in CXCL8 expression via unknown mechanisms which is independent on HIF-1α pathway. CXCL8 secreted from astrocytes may have protective effects on neurons.

Discussion

It has been reported that chelation of iron induces the expression of CXCL8 in human mast cell-derived HMC-1 cells[12], human intestinal epithelial cells[13] and oral keratinocytes[14]. In the present study, we demonstrated that treatment of U373MG astrocytoma cells with desferrioxamine induced the expression of CXCL8, as well as VEGF used as a positive control. We also found that the effect of desferrioxamine on the expression of CXCL8 and VEGF was almost completely abrogated by pretreatment of cells with $FeSO_4$. This confirmed that the effect of desferrioxamine was due to iron chelation.

HIF-1α is a transcriptional factor that functions as a master regulator of the adaptive response to hypoxia, and HIF-1α can be also activated by desferrioxamine[15]. In the present study, knockdown of HIF-1α decreased the expression of VEGF mRNA, but not of CXCL8 mRNA, induced by desferrioxamine. This suggests that HIF-1α is involved in VEGF mRNA expression induced by desferrioxamine, while desferrioxamine induces CXCL8 in a HIF-1α-independent manner (Fig. 4). It has been reported that desferrioxamine activates

NF-κB[16] and p38 MAPK[17]. In an experimental animal model, intranasal desferrioxamine attenuates synapse loss via up-regulating the p38 in experimental animals[17]. Therefore, we examined if NF-κB or p38 MAPK is involved in desferrioxamine-induced CXCL8 expression in U373MG cells. However, knockdown of NF-κB p65 did not affect the CXCL8 expression induced by desferrioxamine, and treatment of cells with desferrioxamine did not induce the phosphorylation of p38 MAPK (data not shown). The mechanisms by which desferrioxamine induced CXCL8 expression have not been clarified in the present study, and it should be investigated in future studies.

CXCL8 was reported to have an activity to protect neurons from amyloid-β-induced neurotoxicity[6], and in the present study, we found that desferrioxamine treatment of U373MG cells increased the expression of CXCL8. Therefore, iron chelation may be helpful to prevent amyloid-β-induced neurotoxicity in Alzheimer's disease at least partly via inducing CXCL8 in addition to via reducing oxidative stress. Crapper McLachlan and colleagues[18] showed a substantial reduction in the rate of deterioration of daily living skills in 48 patients with Alzheimer's disease who were given desferrioxamine when compared with patients with Alzheimer's disease given placebo. Despite such positive results, no other clinical studies of desferrioxmaine have been reported because desferrioxamine has harmful side effects such as dysfunctions of liver and kidney [1]. A previous study reported that intranasal desferrioxamine attenuates synapse loss in the brain of amyloid-β precursor protein and presenilin-1 double transgenic mice used as an animal model of Alzheimer's disease[17]. Development of some stratagies to chelate iron with less side effects may lead to a new therapeutic approach to Alzheimer's disease.

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