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Full Length Article

Clioquinol increases the expression of interleukin-8 by down-regulating GATA-2 and GATA-3

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ABSTRACT

Clioquinol was used in the mid-1900s as an amebicide to treat indigestion and diarrhea. However, it was withdrawn from the market in Japan because it was linked to subacute myelo-optic neuropathy (SMON). The pathogenesis of SMON has not yet been elucidated in detail. As reported previously, we performed a global analysis on human neuroblastoma cells using DNA chips. The global analysis and quantitative PCR demonstrated that the mRNA level of interleukin-8 (IL-8) was significantly increased when SH-SY5Y neuroblastoma cells were treated with clioquinol. An enzyme-linked immunosorbent assay also demonstrated that clioquinol induced the secretion of IL-8 into culture media. Promoter analyses on SH-SY5Y cells revealed that a region responsive to clioquinol exists between –152 and –144 of the human *IL-8* gene, which contains a consensus GATA-binding site sequence. The introduction of mutations at this site or the activator protein (AP)-1 site sequence at –126/–120 significantly reduced clioquinol-induced transcriptional activation. Among the GATA transcription factors expressed in SH-SY5Y cells, GATA-2 and GATA-3 protein levels were significantly decreased by the addition of clioquinol. Electrophoresis mobility shift assays using a probe corresponding to –159/–113 of the human *IL-8* gene revealed two major shifted bands, one of which was increased and the other was decreased by clioquinol. The introduction of mutations showed that the former corresponded to binding to the AP-1 site, and the latter to binding to the GATA site. Supershift analyses revealed that the binding of c-Jun and c-Fos was increased, whereas that of GATA-3 was decreased by clioquinol. Genome editing against GATA-2 or GATA-3, not GATA-4 significantly enhanced clioquinol-induced IL-8 mRNA expression. On the other hand, the stable expression of GATA-2 or GATA-3 attenuated clioquinol-induced IL-8 mRNA expression and IL-8 secretion. These results suggest that the clioquinol-induced suppression of GATA-2 and GATA-3 expression mediates the up-regulation of IL-8.

1. Introduction

Clioquinol (5-chloro-7-indo-8-quinolinol), a $Cu^{2+}/Zn^{2+}/Fe^{2+}$ chelator/ionophore, was used extensively as an amebicide to treat indigestion and diarrhea in the mid-1900s. However, it was withdrawn from the market in Japan because its use was epidemiologically linked to an increase in the incidence of subacute myelo-optic neuropathy (SMON) (Cahoon, 2009; Konagaya, 2015). SMON is characterized by the subacute onset of sensory and motor disturbances in the lower extremities with occasional visual impairments, which are preceded by abdominal symptoms (Nakae et al., 1973; Tsubaki et al., 1971). Although pathological studies demonstrated axonopathy of the spinal cord and optic nerves (Tateishi, 2000), the underlying mechanisms of clioquinol toxicity have yet to be elucidated in detail.

Clioquinol was found to suppress nerve growth factor-induced Trk autophosphorylation and neurite outgrowth in PC12 cells at 1 μM (Asakura et al., 2009). This neuronal damage was shown to be mediated by histone deacetylation (Fukui et al., 2015). Other studies demonstrated that the cytotoxicity of clioquinol was mediated by oxidative stress in murine cortical cultures at 1 μM (Benvenisti-Zarom et al., 2005) and in human neuroblastoma SH-SY5Y cells at 25–50 μM (Kawamura et al., 2014). Involvement of inhibition of the 20S proteasome in leukemia and myeloma cells at 15–25 μM (Mao et al., 2009), or relocation of the cytoplasmic X-linked inhibitor of apoptosis protein to the nucleus in prostate cell lines at 10 μM (Cater and Haupt, 2011) was documented as well.

In order to clarify the molecular mechanisms underlying clioquinol-induced neurotoxicity, we performed a global analysis on human

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neuroblastoma cells using DNA chips (GEO database accession code: GSE32173) and demonstrated that clioquinol at 10–50 μM induced DNA double-strand breaks, leading to the activation of ATM and downstream p53 signaling (Katsuyama et al., 2012). This pathway may, at least partly, contribute to clioquinol-induced neurotoxicity. We also found that clioquinol at 10–50 μM induced the expression of VGF, the precursor of neuropeptides involved in pain reactions, by inducing c-Fos, one of the activator protein (AP)-1 transcription factors (Katsuyama et al., 2014). The induction of VGF suggests its involvement in clioquinol-induced mechanical hyperalgesia and cold allodynia (Andersson et al., 2009).

In the global analysis, we found that the expression of interleukin-8 (IL-8), a key chemokine responsible for the activation and recruitment of neutrophils to sites of inflammation (Baggiolini et al., 1989), was markedly increased by clioquinol. In rat models of nerve damage-induced neuropathic pain, it was reported that IL-8 levels were increased in the injured sciatic nerve and dorsal root ganglion (Khan et al., 2017), suggesting its involvement in pain reactions in SMON. This prompted us to undertake an investigation on the molecular basis of clioquinol-induced IL-8 expression. Promoter analyses suggested the involvement of GATA transcription factors that has never been reported in the regulation of IL-8 expression. Among GATA transcription factors, GATA-2 and GATA-3 were reported to be essential for neurogenesis (Nardelli et al., 1999). GATA-3 was also reported to be involved in the expression of enzymes essential for noradrenaline synthesis (Lim et al., 2000; van Doorninck et al., 1999). In the global analysis, the expression level of GATA-3 was markedly suppressed by clioquinol (Katsuyama et al., 2012), suggesting that the down-regulation of GATA-3 by clioquinol may affect essential neural functions involving IL-8.

We herein report that clioquinol-induced IL-8 expression was mediated by the down-regulation of GATA-2 and GATA-3, a novel pathway involved in the regulation of IL-8 expression.

2. Materials and methods

2.1. Materials

Clioquinol was purchased from Merck (Darmstadt, Germany). Antibodies against GATA-2 [EPR2822(2)], GATA-3 [EPR16651], and GATA-4 [EPR4768] were purchased from Abcam (Cambridge, UK). Antibodies against c-Jun (60A8) and c-Fos (9F6) were purchased from Cell Signaling Technology (Danvers, MA). The antibody against c-Jun used in the electrophoresis mobility shift assay (EMSA) was obtained from Active Motif (Carlsbad, CA). The antibody against β-actin (6D1) was obtained from Medical & Biological Laboratories (Nagoya, Japan). [γ-^{32}P]-ATP was purchased from PerkinElmer (Waltham, MA).

2.2. Cell culture

Human neuroblastoma SH-SY5Y cells, often used as an in vitro model for studying neurotoxicity (Cheung et al., 2009; Xie et al., 2010), were purchased from the European Collection of Cell Cultures and cultured in Ham's F12: Eagle's medium with Earle's salts (1:1) supplemented with non-essential amino acids and 15% fetal bovine serum (FBS). Clioquinol dissolved in DMSO was added to the culture medium at 1/1000 v/v. As a control, DMSO was added at 1/1000 v/v. Cells were stimulated for 24 h with 0, 10, 20, or 50 μM clioquinol for dose dependency experiments, and with 50 μM clioquinol for 0, 3, 8, or 24 h for time course experiments. Cell viability at 24 h of clioquinol treatment was examined using a CyQuant Direct Cell Proliferation Assay Kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions, as described previously (Katsuyama et al., 2012).

2.3. Quantitative PCR

Quantitative PCR was performed as described previously (Katsuyama et al., 2012). Cells were seeded on 6-well plates (5×10^5 cells/well), cultured for 24 h, and then stimulated with the indicated concentration of clioquinol for 24 h (dose dependency) or with 50 μM clioquinol for the indicated time (time course). Gene expression was quantified using standard curves that were generated using serially diluted plasmid reference samples and normalized to the expression level of hypoxanthine phosphoribosyltransferase (HPRT). The specificities of the PCR products were confirmed by gel electrophoresis and a dissociation curve analysis. The sequences of primers for the detection of IL-8 were as follows: 5′-ATTTCTGCAGCTCTGTGTGAAGGT-3′ for sense, 5′-TTTTTTATGAATTCTCAGCCCTCT-3′ for antisense. Following primers were used for the detection of HPRT as described previously (Katsuyama et al., 2012): 5′-AGACTTTGCTTTCCTTGGTCA-3′ for sense, 5′-AGGCTTTGTATTTTGCTTTTC-3′ for antisense.

2.4. Measurement of IL-8 levels in culture media

Cells were seeded on 24-well plates (1×10^5 cells/well) and cultured for 24 h. Then the media were replaced with 500 μl of new media containing the indicated concentration of clioquinol (50 μM for time course), and cells were incubated for the indicated time (24 h for dose dependency). IL-8 levels secreted in media were measured using the Quantikine ELISA Human IL-8/CXCL8 Immunoassay kit according to the manufacturer's protocol (R&D Systems, Minneapolis, MN). An aliquot of media (50 μl) was used for the determination of IL-8 concentration. In cases that exceed the maximum concentration of the standard curve (2000 pg/ml), media were diluted (x10) before the measurement to fit the standard curve, and the dilution ratio was reflected to the data.

2.5. Reporter constructs and luciferase assay

Human genomic DNA was isolated from SH-SY5Y cells with the PUREGENE DNA Isolation Kit (Gentra SYSTEMS, Minneapolis, MN). The 5′-flanking and non-coding regions of the human *IL-8* gene were amplified by PCR using KOD-Plus- (TOYOBO, Osaka, Japan) and cloned into the NanoLuc vector pNL1.2 (Promega, Madison, WI). The 1.5-kb 5′-flanking and non-coding regions were cloned into the EcoRV/HindIII site of pNL1.2. A series of 5′-deletion constructs were made by amplification using PCR. All constructs were subjected to sequencing analyses in order to verify the orientation and fidelity of the insert. SH-SY5Y cells were seeded on 24-well plates at 1×10^5 cells/well and grown for 24 h, and NanoLuc plasmids (0.4 μg/well) and the firefly luciferase vector pGL4.54 (0.1 μg/well; Promega, Madison, WI) were co-transfected with TransIT-LT1 Reagent (Mirus Bio, Madison, WI). These cells were cultured for 24 h and then stimulated with 50 μM clioquinol for 24 h. NanoLuc activity in cell lysates was assessed and normalized with firefly luciferase activity.

2.6. Western blotting

Cells were seeded on 6-well plates (5×10^5 cells/well), cultured for 24 h, and then stimulated with 50 μM clioquinol for 24 h. Whole cell lysates were prepared as described previously (Katsuyama et al., 2012). Briefly, cells were washed twice with phosphate-buffered saline and lysed in a buffer containing 1% Triton, 0.5% sodium deoxycholate, 10 mM TrisHCl (pH 6.8), 150 mM NaCl, 1 mM EDTA, protease inhibitor cocktails (Nacalai Tesque, Kyoto, Japan), 1 mM NaF, 20 mM β-glycerophosphate, and 1 mM Na_3VO_4. The lysate was centrifuged and the supernatant was used as whole cell lysate. Aliquots containing equal amounts of protein (10 μg) were separated by handmade 10% SDS-polyacrylamide gels with a molecular size marker (Precision Plus Dual Standard, Bio-Rad, Tokyo, Japan), and transferred onto polyvinylidene