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Research article

Luteolin attenuates interleukin-6-mediated astrogliosis in human iPSC-derived neural aggregates: A candidate preventive substance for maternal immune activation-induced abnormalities

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HIGHLIGHTS

- Human iPSC system reflecting the "neuron-first, glia-second" differentiation pattern.
- IL6 increases astrocyte and decreased neuron density in this human iPSC-based system.
- Luteolin attenuates IL6-mediated neural changes in human iPSC-derived aggregates.

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ABSTRACT

Maternal infection during pregnancy increases the risk of neurodevelopmental conditions such as autism spectrum disorders and schizophrenia in offspring. Several previous animal studies have indicated that maternal immune activation (MIA), rather than a specific pathogen, alters fetal brain development. Among them, prenatal exposure to interleukin-6 (IL-6) has been associated with behavioral and neuropathological abnormalities, though such findings remain to be elucidated in humans. We developed a human cell-based model of MIA by exposing human induced pluripotent stem cells (hiPSCs)-derived neural aggregates to IL-6 and investigated whether luteolin—a naturally occurring flavonoid found in edible plants—could prevent MIA-induced abnormalities. We generated neural aggregates from hiPSCs using the serum-free floating culture of embryoid body-like aggregates with quick reaggregation (SFEBq) method, following which aggregates were cultured in suspension. We then exposed the aggregates to IL-6 (100 ng/ml) for 24 h at day 51. Transient IL-6 exposure significantly increased the area ratio of astrocytes (GFAP-positive area ratio) and decreased the area ratio of early-born neurons (TBR1-positive or CTIP2-positive area ratio) relative to controls. In addition, western blot analysis revealed that levels of phosphorylated STAT3 were significantly elevated in IL-6-exposed neural aggregates. Luteolin treatment inhibited STAT3 phosphorylation and counteracted IL-6-mediated increases of GFAP-positive cells and reductions of TBR1-positive and CTIP2-positive cells. Our observations suggest that the flavonoid luteolin may attenuate or prevent MIA-induced neural abnormalities. As we observed increased apoptosis at high concentrations of luteolin, further studies are required to determine the optimal intake dosage and duration for pregnant women.

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1. Introduction

The development of central nervous system (CNS) comprises many highly orchestrated steps that can be impaired by various types of environmental stress [1,2]. Maternal infections during pregnancy are well known to affect fetal CNS development, and contribute to neurodevelopmental disorders such as autism spec-

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trum disorders (ASD) and schizophrenia in offspring [2,3]. Although many epidemiological studies have shown that increased incidence of ASD and schizophrenia are associated with exposure to prenatal viral and bacterial infections [4–7], several studies of rodents have revealed that maternal immune activation (MIA), rather than specific pathogens themselves, plays a key role in influencing fetal brain development. Studies have suggested that elevated levels of interleukin-6 (IL-6) are significantly associated with MIA-induced neurodevelopmental abnormalities [8,9]. However, despite their advantages in experimental tractability, rodent models often do not accurately reflect human pathophysiology. Owing to ethical issues and the inaccessibility of human neural tissue, our current understanding of MIA-induced effects on neurodevelopment in humans remain extremely limited, though a few studies using non-human primates have been published [10–12]. Hence, there are no available strategies for the prevention of MIA-induced neurodevelopmental disorders.

Human induced pluripotent stem cells (hiPSCs) and their differentiation into neural cells represent powerful tools for investigating the mechanisms associated with the pathogenesis of human brain disorders, especially disease-associated alterations occurring at relatively early or fetal stages. Furthermore, they provide a novel platform for evaluating drug candidates. To our knowledge, no studies have utilized hiPSC-derived neural cells as a model for MIA to date. Therefore, in the present study, we investigated histopathological changes in neural aggregates derived from healthy human iPSCs following exposure to IL-6. Furthermore, we evaluated whether luteolin,

a naturally occurring flavonoid found in edible plants, could prevent IL6-mediated histopathological alterations in these aggregates.

2. Materials and methods

2.1. Neural differentiation of hiPSCs

The hiPSC line (409B2) was provided by the RIKEN BRC through the Project for Regenerative Medicine and the National Bio-Resource Project of the MEXT, Japan. Ethical approval for this study was obtained from the Review Board of Kyoto Prefectural University of Medicine. Neural differentiation was performed as previously described [13]. Briefly, the iPSCs were dissociated to single cells and quickly reaggregated in 96-well low cell-adhesion plates. Aggregates were cultured in suspension for the first 20 days in neural induction medium (DMEM/F12 medium supplemented with KSR, NEAA, 2-mercaptoethanol, L-glutamate, SB431542, Dorsomorphin, and Y27632). Neural aggregates were then cultured in neural differentiation medium (DMEM/F12 medium and neurobasal medium supplemented with the 0.5% N2, 1% B27 and 2 mM L-glutamine) beginning at day 20 (Fig. 1A). The medium was changed every 3–4 days.

To examine the neural impact of IL-6 exposure, neural aggregates were cultured with recombinant human IL-6 (100 ng/ml from PeproTech Inc., Rocky Hill, NJ) in the presence or absence of various concentrations of luteolin (10, 20, 40, 80 μM, from Sigma-Aldrich Japan, Tokyo, Japan) or the signal transducer and activator of transcription-3 (STAT3)-specific inhibitor Stattic (10 μM, from Tocris Bioscience, Bristol, UK) at day 51 (Fig. 1A). Media were changed 24 h after IL-6 exposure, and aggregates were cultured in suspension to day 60 for immunohistochemical analysis. To examine the size of neural aggregates, the area of the aggregates in each image was measured using ImageJ software.

2.2. Reverse transcription-polymerase chain reaction

Total RNA extraction from cells was performed using the RNeasy Mini kit (QIAGEN, Venlo, Netherlands) according to the manufacturer's protocols. The Prime Script RT reagent Kit (Takara Bio, Otsu, Japan) was used to synthesize cDNA according to the manufacturer's protocols, and qRT-PCR was performed using the 7500 Real-time PCR System (Life Technologies, Carlsbad, CA) with SYBR Premix Ex Taq (Takara Bio). The primer sequences are shown in Table S1.

2.3. Western blot analysis

Neural aggregates were lysed in RIPA buffer following IL-6 exposure in the presence or absence of luteolin or Stattic for 12 h at day 51. Equal amounts of total protein (10 μg/lane) were diluted in NuPAGE sample buffer and separated in an Xcell SureLock electrophoresis unit with NuPAGE 10% bis-tris gels in NuPAGE MOPS buffer according to manufacturer's instructions (Life Technologies), following which they were electrophoretically transferred onto polyvinylidene fluoride membranes (GE Healthcare, Milwaukee, WI) using an Xcell Blot Module (Life Technologies). After blocking in TBS containing 0.1% Tween-20 (TBS-T) and 5% fat-free dry milk for 1 h, the membranes were then incubated overnight in primary antibodies targeting phosphorylated-STAT3 (1:2000, Cell Signaling Technology, Danvers, MA), STAT3 (1:2000, Cell Signaling Technology), cleaved-caspase 3 (1:1000, Cell Signaling Technology), and β-actin (1:5000, Sigma Aldrich), followed by the appropriate secondary antibodies for 1 h. The membranes were then visualized using ECL or ECL prime chemiluminescence reagents (GE Healthcare). Three independent experiments were performed.

2.4. Immunohistochemistry

Neural aggregates were fixed in 4% paraformaldehyde for 20 min at 4 °C, then soaked in 30% sucrose, and maintained at 4 °C for 48–72 h. Subsequently, the aggregates were transferred into O.C.T Compound 4583 and cryosectioned at 20 μm. For immunohistochemistry, sections were blocked in 0.1% bovine serum albumin (BSA) and PBS containing 0.3% Triton X-100 (PBS-T) for 1 h at room temperature. The sections were incubated overnight with primary antibodies in PBS-T and then labeled with fluorescent-tagged secondary antibodies for 1 h. The following primary and secondary antibodies were used in this assay: CTIP2 (1:1000, Abcam, Cambridge, UK), TBR1 (1:200, Abcam), SATB2 (1:500, Abcam), GFAP (1:1000, Abcam). To quantify the immunohistochemistry analysis, we calculated the average of the area ratio for each antibody (primary antibody-positive area/whole neural aggregate area) using BIOREVO BZ-x710 and BZX Analyzer (Keyence, Osaka, Japan).

2.5. Statistical analysis

All data are presented as mean ± SD. Pairs of groups were analyzed using an unpaired Student's *t*-test. $P < 0.05$ was considered statistically significant. We performed analyses using Excel Tokei (Social Survey Research Information, Tokyo, Japan).

3. Results

3.1. Neural aggregates reflect the time course of fetal brain development

To determine whether our neural aggregates accurately reflected fetal cortical differentiation, we examined the temporal mRNA expression of neural markers. First, expression of PAX6 (a marker for neural stem cells) was upregulated, following which

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