主論文の要旨

Interleukin-1β Induces Blood-Brain Barrier Disruption by Downregulating Sonic Hedgehog in Astrocytes

（インターロイキン-1βはアストロサイトのソニックヘッジホッグの発現低下を介して血液脳関門の破綻を引き起こす）

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<Background>

The blood–brain barrier (BBB) is a tight seal composed of capillary endothelial cells, pericytes, and perivascular astrocytes. The BBB contributes to homeostasis in the central nervous system (CNS) by limiting the entry of plasma components, erythrocytes, and immune cells from the circulating blood. Astrocytes play a pivotal role in maintenance of BBB integrity via contact-dependent mechanisms and release of trophic factors. In addition, a recent study revealed that Sonic hedgehog (SHH) released from astrocytes promotes BBB formation and integrity by upregulating tight junction (TJ) proteins in capillary endothelial cells. Without SHH, its receptor Patched-1 (Ptch-1) suppresses a G-coupled–protein receptor Smoothened (Smo) which is critical for the activation of a transcription factor Gli-1. Gli-1 is an important regulator of TJ protein expression and BBB formation. SHH binds and inactivates Ptch-1, which allows Smo to activate Gli-1, which upregulates TJ proteins and enhances BBB integrity. Disruption of BBB integrity is frequently observed in neurologic diseases such as multiple sclerosis (MS), Parkinson's disease, amyotrophic lateral sclerosis, and Alzheimer's disease, suggesting that infiltrating molecules and immune cells from the blood perturb CNS homeostasis and exacerbate these disorders. Microglial activation is another characteristic pathologic feature in these diseases. Activated microglia release various cytotoxic factors such as nucleic acids, glutamate, reactive oxygen species (ROS), proteases, and pro-inflammatory cytokines/chemokines. Interleukin-1β (IL-1β) is a major microglial pro-inflammatory cytokine that acts on both endothelial cells and astrocytes to increase BBB permeability. However, the mechanisms of BBB disruption by IL-1β have not been fully elucidated. In this study, we demonstrated that IL-1β suppressed SHH expression in astrocytes and increased BBB permeability by downregulating TJ proteins in endothelial cells. Moreover, IL-1β stimulated astrocytes to secrete pro-inflammatory chemokines such as CCL2, CCL20, and CXCL2, which induce the migration of immune cells such as neutrophils, monocytes, macrophage, dendritic cells, and pathogenic T cells. Our findings reveal novel mechanisms of BBB disruption by IL-1β, and suggest that SHH could be used therapeutically against various neurologic diseases.

<Materials and methods>

Mouse primary astrocytes were prepared from newborn C57BL/6J mice. Confluent monolayers of MBEC4 (mouse brain capillary endothelial cell line) on Transwell inserts were used as an *in vitro* BBB model. The mRNA expression levels were assessed by quantitative PCR. The protein expression levels were measured by Western blotting and ELISAs.

<Results>

IL-1β suppressed the protective effect of astrocytes on BBB integrity.

We confirmed the effects of IL-1β and astrocytes on BBB integrity using MBEC4 monolayers as an *in vitro* BBB model. Astrocyte conditioned media (ACM) significantly

decreased the permeability of BBB (Fig. 1). Treatment with IL-1β alone significantly increased the permeability of BBB, and conditioned media from IL-1β–stimulated astrocytes lost the ability to increase BBB integrity (Fig. 1). These findings suggested that IL-1β disrupts BBB integrity not only directly, but also indirectly via astrocyte dysfunction.

IL-1β decreased astrocytic production of SHH.

Next, we focused on SHH, a soluble factor released from astrocytes that plays an important role in BBB maintenance. Specifically, we investigated whether IL-1β affects astrocytic SHH expression. Treatment with IL-1β significantly decreased *Shh* mRNA levels in astrocytes in a dose-dependent manner (Fig. 2A). Similar results were obtained for SHH protein levels in ACM using specific ELISA (Fig. 2B).

SHH produced by astrocytes is critical for maintenance of BBB integrity by upregulating tight junction proteins.

Next, we examined the effect of astrocytic SHH signaling on BBB function. SHH or the Smo agonist (i.e. a SHH signaling enhancer) purmorphamine significantly decreased BBB permeability (Fig. 3A). By contrast, the Smo antagonist (i.e. a SHH signaling inhibitor) cyclopamine abolished the astrocytic effect on the maintenance of BBB function (Fig. 3B). The expression levels of such TJ proteins as claudin-5, occludin, and ZO-1 were closely correlated with BBB integrity (Fig. 4A–C): levels of these proteins were highest when permeability was lowest. Activation of SHH signaling by ACM, SHH, or purmorphamine resulted in significant upregulation of these proteins, whereas the Smo antagonist cyclopamine ablated the astrocytic effect on their expression (Fig. 4A–C). These observations suggested that SHH produced by astrocytes plays a critical role in BBB integrity by upregulating expression of TJ proteins.

IL-1β stimulated pro-inflammatory chemokine production in astrocytes.

Finally, we assessed the effects of IL-1β on the production of pro-inflammatory chemokines in astrocytes. Treatment with IL-1β significantly increased the mRNA and protein expression levels of CXCL2, CCL2, and CCL20 in astrocytes (Fig. 5A and 5B). These data imply that IL-1β also activates astrocytes to release these pro-inflammatory chemokines; induces migration of immune cells such as neutrophils, monocytes, macrophage, dendritic cells, and pathogenic T cells; and leads to further BBB disruption and neuroinflammation.

<Discussion>

Here, we propose another novel mechanism for IL-1β–mediated BBB disruption. SHH is a critical activator of Smo–Gli-1 signaling which upregulates TJ proteins and enhances BBB integrity (Fig. 6A). A decrease in SHH allows Ptch-1 to suppress Smo–Gli-1 signaling. In the healthy state, astrocytes release SHH, which upregulates TJ proteins in endothelial cells and maintains BBB integrity (Fig. 6B, left). Once pathogenic stimuli activate microglia to release IL-1β (Fig. 6B, right), it suppresses SHH production in astrocytes, downregulates TJ proteins in endothelial cells, and disrupts BBB integrity. Moreover, IL-1β–stimulated astrocytes secrete the

pro-inflammatory chemokines CXCL2, CCL2, and CCL20, which induce migration of immune cells such as neutrophils, monocytes, macrophage, dendritic cells, and pathogenic T cells. Infiltration of these cells exacerbates BBB disintegrity and subsequent neuroinflammation.

In this study, the Smo antagonist cyclopamine decreased TJ protein expression levels and BBB integrity exceeding the physiological levels (Figs. 3 and 4). Previous reports suggested that unidentified endogenous ligands of Smo seem to activate this signaling although SHH is the main regulator of Smo–Gli-1signaling. Our data also imply the presence of endogenous ligand(s) of Smo. BBB disruption is a common pathologic feature of neurologic disorders such as stroke, MS, Parkinson's disease, amyotrophic lateral sclerosis, and Alzheimer's disease. Therefore, restoration of BBB integrity has been recognized as a therapeutic target for treatment of these diseases. In fact, both glucocorticoids and interferon β, both of which have been widely used for MS treatment, decrease BBB permeability. Moreover, the efficacy of the α4-integrin antagonist natalizumab has also demonstrated the utility of BBB-targeting drugs in treating MS. By contrast, excessive immunosuppression resulting from conventional therapies for MS sometimes causes progressive multifocal leukoencephalopathy. Thus, from the perspective of adverse effects, restoration of TJ proteins represents a superior therapeutic approach. Inhibition of IL-1β is a promising potential method for restoring BBB integrity; however, a previous study indicated that simple blockade of IL-1β runs the risk of increasing BBB disruption, because this cytokine also enhances the protective effects of astrocytes on the BBB. Treatment with SHH may circumvent this dilemma, allowing reinforcement of BBB integrity without loss of the beneficial effects of IL-1β.

<Conclusion>

This study reveals a novel mechanism for IL-1β–mediated BBB disruption: downregulation of SHH expression in astrocytes. Our findings suggest that stimulation of astrocytic SHH production could promote restoration of BBB integrity, and may therefore be useful in treating a variety of neurologic disorders.