Pivotal Role of Signal-Transducing Adaptor Protein-2 in Pathogenesis of Autoimmune Hepatitis

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Signal-transducing adaptor protein-2 (STAP-2) is an adaptor protein involved in inflammatory and immune responses, such as inflammatory bowel disease and allergic responses. In this study, we investigated the role of STAP-2 in the pathogenesis of autoimmune hepatitis. After intravenous injection of concanavalin A (ConA), STAP-2 knock out (KO) mice showed more severe liver necrosis along with substantial lymphocyte infiltration compared to wild type (WT) mice. Serum alanine aminotransferase levels were significantly higher in ConA-injected STAP-2 KO mice than in WT mice. Levels of interferon-γ (IFN-γ), an important factor for liver necrosis, were also significantly increased in sera of STAP-2 KO mice compared to WT mice after ConA injection. Statistically significant upregulation of Fas ligand (FasL) expression was observed in the livers of ConA-injected STAP-2 KO mice compared to WT mice. In accordance with these results, apoptotic signals were facilitated in STAP-2 KO mice compared to WT mice after ConA injection. Correctively, these results suggest that STAP-2 is involved in the pathogenesis of autoimmune hepatitis by regulating the expression of FasL and the production of IFN-γ.

Key words signal-transducing adaptor protein-2; autoimmune hepatitis; interferon-γ (IFN-γ); Fas ligand (FasL); caspase-3

INTRODUCTION

The signal-transducing adaptor protein (STAP) family consists of two members, STAP-1 and STAP-2. In a previous study, we identified the murine STAP-2 sequence and reported that STAP-2 binds to signal transducer and activator of transcription 3 (STAT3) through its YXXQ motif in the proline-rich region to regulate STAT3 activity. STAP-2 is expressed in a number of immune cells, such as T cells, macrophages and mast cells/basophils, and also participates in inflammatory immunological responses, such as stromal cell-derived factor (SDF)-1α-induced chemotaxis, toll-like receptor (TLR)-mediated proinflammatory cytokine production, and FceRI-dependent allergic inflammatory reactions.

Autoimmune hepatitis (AIH) is a severe autoimmune disorder characterized by abundant autoantibody production, increased levels of alanine aminotransferase (ALT), increased hepatocyte apoptosis, and a high number of infiltrated cells in the liver. Because no curative therapies are available, the QOL is very low in patients with AIH. Therefore, there is an urgent need to identify promising targets so as to develop new curative therapies.

In this regard, T cell and invariant NKT (iNKT) cell are essential for development of concanavalin A (ConA)-induced AIH mouse model. In this study, we investigated the involvement of STAP-2, which is a crucial regulator of immune and inflammatory responses, in the onset and development of AIH.

MATERIALS AND METHODS

Mice C57BL/6 mice were purchased from SANKYO LABO SERVICE CO., Inc. (Hokkaido, Japan). STAP-2 knock out (KO) mice were previously generated. All animal studies were approved by the Hokkaido University animal ethics committee. All mice were housed and bred in the Pharmaceutical Sciences Animal Center of Hokkaido University under specific pathogen-free conditions.

Antibodies Fluorescein isothiocyanate (FITC) anti-TCR/β and PE anti-mouse NK1.1 monoclonal antibodies (mAbs) were purchased from Cell Signaling Technology (Beverly, MA, U.S.A.). Anti-STAP-2 and anti-β-actin mAbs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Anti-cleaved caspase-3 Ab was purchased from Cell Signaling Technology (Beverly, MA, U.S.A.).

Hepatitis Mouse Model The age-matched male/female mice were intravenously injected with ConA (15 mg/kg, Sigma-Aldrich, St. Louis, MO, U.S.A.). Plasma ALT levels were measured using SRL service. Interferon-γ (IFN-γ) levels in supernatants were measured using enzyme-linked immunosorbent assay (ELISA) kits (BioLegend). Formalin-fixed paraffin-embedded liver sample specimens (5 μm) were stained with hematoxylin and eosin. Necrotic areas in the livers were measured using ImageJ program (NIH, Bethesda, MD, U.S.A.).

Immunohistochemistry Liver homogenates were prepared using cold phosphate buffered saline (PBS) containing 1 mM phenylmethylsulfonyl fluoride (PMSF). Immunohistochemistry analysis was performed as previously described. Actin was detected as a loading control.

Flowcytometric Analysis Flowcytometric analysis was performed as previously described.
performed as previously described.6)

**Quantitative (q)PCR** QPCR was performed as previously described.6) PCR primer sequences are shown as follows; Il4 (forward: CATCGGACATTTTGAAAGCAG, reverse: CGAGCTCCTCTGGTGTTG), Fasl (forward: ACCGGTGTTATTTCATGG, reverse: TTTAGGCTTGGTTGTGAAA), Gzmb (forward: GCTGCTCATTGGTAAAG, reverse: TGAGGATAGCATTTTACCAT), G3pdh (forward: ACCACAAGGCCTCATGCCATAC, reverse: TCCACCACCCTGTGTGGTAG).

**Statistical Analysis** Statistical analysis was performed using GraphPad Prism 6.02. Mann–Whitney U-test was employed. Data were considered significant at p < 0.05. Data were shown mean ± standard error of the mean (S.E.M.).

**RESULTS**

**STAP-2 Is Involved in Controlling the Pathogenesis of ConA-Induced Hepatitis** STAP-2 was expressed in the liver of wild type (WT) mice, but not of STAP-2 KO mice (Fig. 1A), confirming the complete elimination of STAP-2 protein. To investigate the role of STAP-2 in the pathogenesis of AIIH, we first compared morphological changes in the livers of WT and STAP-2 KO mice after intravenous injection of ConA. Morphological features of livers from untreated STAP-2 KO mice were similar to those of untreated WT mice (data not shown). Liver necrosis with lymphocyte infiltration was observed in WT mice injected with ConA (Fig. 1B). A larger area of necrosis and increased lymphocyte infiltration were observed in the livers of ConA-injected STAP-2 KO mice (Fig. 1B). This difference was confirmed by measuring the necrotic area, serum ALT level, and cytokine production. The proportion of necrotic area in ConA-injected STAP-2 KO mice was significantly higher than that in WT mice (Fig. 1C).

Next measured levels of ALT, IFN-γ and interleukin (IL)-4 in ConA-administered WT and STAP-2 KO mice. These levels of untreated STAP-2 KO mice were comparable to those of untreated WT mice. In ConA-administered STAP-2 KO mice, serum ALT and IFN-γ levels were significantly higher compared to WT mice. We also observed increased Il4 expression in ConA-administered STAP-2 KO mice compared to WT mice although no statistical significance (Figs. 1D–F). These results suggest that STAP-2 KO mice are more susceptible to ConA-induced hepatitis than WT mice.

**Aberrant Apoptosis Signaling in ConA-Injected STAP-2 KO Mice** The proportions of NK cells, but not T cells or iNKT cells, in lymphocytes invading the liver after ConA injection were significantly higher in STAP-2 KO mice than in WT mice while the proportions were same between untreated WT and STAP-2 KO mice (Fig. 2A). Among cell death-related genes, expression of Fasl, but not Gzmb, was significantly higher in the livers of ConA-injected STAP-2 KO mice than those of WT mice while expression levels of both genes were same between untreated WT and STAP-2 KO mice (Fig. 2B). We also evaluated the expression of other genes, such as those encoding perforin, IL-6, and tumor necrosis factor (TNF)-α; no differences in their gene expression were observed (data not shown).

On comparing the levels of cleaved caspase-3 in the livers of both groups, we found that the cleaved caspase-3 levels in WT mice were slightly increased 6h after ConA administration. The levels in ConA-administered STAP-2 KO mice tended to be higher than the levels in ConA-administered WT mice although statistical significance was not observed between groups (Figs. 2C, D). Correctively, these results suggest that STAP-2 is involved in the upregulation of Fas ligand (FasL) expression induced by ConA injection, resulting in enhanced apoptotic signals in hepatocytes.
DISCUSSION

In this study, we demonstrated that the symptoms of ConA-induced hepatitis were more severe in STAP-2 KO mice than in WT mice. STAP-2 KO mice showed higher levels of serum ALT as well as increased IFN-γ and IL-4 production after intravenous injection of ConA, and the proportion of NK cells in the livers was significantly higher in ConA-injected STAP-2 KO mice than in WT mice. In addition, FasL expression in the livers of STAP-2 KO mice after ConA injection was significantly higher than that in WT mice. Taken together, STAP-2-deficiency likely enhanced the apoptotic signal during the onset and development of ConA-injected AIH in this murine model.

AIH is cryptogenic hepatitis characterized by production of high levels of autoantibodies and extensive hepatocyte cell death. Several reports have shown both harmful and protective roles of cytokines in the pathogenesis of AIH. For instance, Reportedly, IFN-γ-deficient mice are resistant to ConA-induced hepatitis, suggesting that IFN-γ signaling is critical for AIH.7) For the pathogenesis of AIH, the Fas/FasL cascade is essential because FasL-deficient gld/gld mice and Fas-deficient lpr/lpr mice are highly resistant to ConA-induced hepatitis.8,9) IL-4 is also critical for ConA-induced hepatitis through the upregulation of Fasl and Gzm b expression.10) IL-6 has a protective role against AIH, and the IL-6–gp130–STAT3 pathway protects against ConA-induced hepatitis.11) In the present study, we found that Fasl levels were significantly increased in the livers of STAP-2 KO mice compared to WT mice after ConA injection. Since we previously reported that STAP-2 positively regulates the expression of Fas, but not FasL, the upregulation of Fasl expression in this model may be indirectly mediated by changes in the environment, including IL-4 and/or IFN-γ production. Alternatively, our previous study showed that IL-6-induced STAT3 phosphorylation in hepatocytes is impaired in the absence of STAP-2,2) whereas STAP-2 directly regulates the IL-6/STAT3/SOCS3 cascade.12) Thus, STAP-2-mediated STAT3 activation may be another reason why STAP-2 suppresses the development of ConA-induced hepatitis.

In conclusion, we demonstrated that STAP-2 KO mice were more susceptible to ConA-induced hepatitis and produced higher levels of IFN-γ/IL-4 and upregulated FasL expression, resulting in augmentation of hepatocyte cell death. Therefore, STAP-2 expression is a key factor in controlling the onset and development of AIH.

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Conflict of Interest The authors declare no conflict of interest.

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