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Original paper

Absence of kynurenine 3-monooxygenase reduces mortality of acute viral myocarditis in mice

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ABSTRACT

Infection of the encephalomyocarditis virus (EMCV) in mice is an established model for viral myocarditis. Previously, we have demonstrated that indoleamine 2,3-dioxygenase (IDO), an L-tryptophan – kynurenine pathway (KP) enzyme, affects acute viral myocarditis. However, the roles of KP metabolites in EMCV infection remain unclear. Kynurenine 3-monooxygenase (KMO) is one of the key regulatory enzymes, which metabolizes kynurenine to 3-hydroxykynurenine in the KP. Therefore, we examined the role of KMO in acute viral infection by comparing between $KMO^{-/-}$ mice and $KMO^{+/+}$ mice. KMO deficiency resulted in suppressed mortality after EMCV infection. The number of infiltrating cells and $F4/80^+$ cells in $KMO^{-/-}$ mice was suppressed compared with those in $KMO^{+/+}$ mice. $KMO^{-/-}$ mice showed significantly increased levels of serum KP metabolites, and induction of KMO expression upon EMCV infection was involved in its effect on mortality through EMCV suppression. Furthermore, $KMO^{-/-}$ mice showed significantly suppression of CCL2, CCL3 and CCL4 on day 2 and CXCL1 on day 4 after infection. These results suggest that increased KP metabolites reduced chemokine production, resulting in suppressed mortality upon KMO knockdown in EMCV infection. KP metabolites may thus provide an effective strategy for treating acute viral myocarditis.

1. Introduction

Encephalomyocarditis virus (EMCV), a member of the Picornaviridae family that includes the Enterovirus genus, can cause acute myocarditis in various animals including mice. EMCV infection in mice is an established model for viral myocarditis, dilated cardiomyopathy, and congestive heart failure [1]. In the early stages of EMCV infection, inflammatory responses are detected in the heart, where high levels of circulating pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6 via NF-κB signaling have been measured [2–5]. After EMCV infection, multiple cytokines and chemokines are produced in macrophages, neutrophils, $CD4^+$ and $CD8^+$ T lymphocytes, and in mast cells [6–8]. The inflammatory response that is induced upon infection plays a critical role in EMCV pathogenesis.

Recently, we demonstrated that the inhibition of indoleamine 2,3-dioxygenase (IDO), which catalyzes the degradation of tryptophan (TRP) to kynurenine (KYN) in the kynurenine pathway (KP), ameliorates EMCV-induced myocarditis [9]. Moreover, it is known that KP regulates inflammation, oxidative stress and immune responses under acute heart attack [10]. Therefore, regulation of KP may provide an effective strategy for treating acute viral myocardi-

Abbreviation: 3-HK, 3-hydroxykynurenine; AA, anthranilic acid; EMCV, encephalomyocarditis virus; IDO, indoleamine 2,3-dioxygenase; KA, kynurenic acid; KMO, kynurenine 3-monooxygenase; KP, kynurenine pathway; KYN, kynurenine; TRP, tryptophan.

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tis. However, the roles of KP metabolites after EMCV infection remain unclear.

It has recently been reported that kynurenine 3-monooxygenase (KMO) deficiency alters the KP [11,12]. KMO is a pivotal enzyme in the KP and normally oxidizes KYN to 3-hydroxykynurenine (3-HK) in the presence of reduced nicotinamide adenosine dinucleotide phosphate (NADPH) and molecular oxygen. KMO localizes to the outer mitochondrial membrane and is mainly expressed in peripheral tissues such as the kidney, liver, spleen, and lungs, and in phagocytes such as macrophages [13,14]. Along with others, we have shown that pro-inflammatory cytokines including IFN-γ stimulate KMO activity both in the periphery and in the brain, when the immune system is activated [15–17]. Previous studies have shown that KMO inhibition in blood ameliorates neurodegenerative disorders such as Alzheimer's disease and Huntington's disease [18–21]. Furthermore, a most recent report suggested KMO inhibition as a novel therapeutic strategy in the treatment of acute pancreatitis-induced multi-organ dysfunction syndrome [12]. Therefore, KMO is suggested to play a major role in physiological and pathological events through the KP.

In this study, the role of KP metabolites in immune regulation was examined by acute viral infection of EMCV in $KMO^{-/-}$ mice. We showed that increased levels of serum KP metabolites suppressed the production of various chemokines by preventing the recruitment of immune cells in local infection, resulting in the reduction of EMCV-infected cells and mortality.

2. Material and methods

2.1. Mice

Male mice at 6-8–weeks- of age were used in this study. KMO gene-deficient ($KMO^{-/-}$) mice on a C57BL/6J background were obtained from the Knockout Mouse Project (KOMP) repository. Homozygous $KMO^{-/-}$ and $KMO^{+/+}$ mice were generated by intercrossing heterozygous ($KMO^{+/-}$) mice. All the mouse genotypes were confirmed by standard PCR-based genotyping of genomic DNA extracted from tail snippets. The following primer sequences were used for PCR genotyping: KMO gene sense: 5′-TTCTGACCCCATCTGTGTCTGTTCC-3′, antisense: 5′-ATCAGAGCTCCCTAAATATGGTGGC-3′, KMO gene deficiency sense: 5′-AACTTCGACCCTTTCCCAC-3′, antisense: 5′-GACCACCTCATCAGAGCAG-3′. The mice were housed in a specific pathogen-free environment within our animal facility prior to use. All experiments were performed in accordance with the Guidelines for Animal Care of the Kyoto University.

2.2. Virus infection

A myocarditic variant of EMCV was generously provided by Dr. Y. Seto (Keio University, Tokyo, Japan). The virus stock was stored at −80 °C in HBSS with 0.1% BSA until use. Virus infection was performed as described previously [9,22]. Briefly, the mice were injected intraperitoneally with 500 PFU EMCV in 0.2 ml saline and then housed in an isolated room. Mortality after EMCV infection was monitored in $KMO^{-/-}$ mice (n = 27) and in $KMO^{+/+}$ mice (n = 27). To obtain samples, the animals were anesthetized and humanely sacrificed at the indicated times. The day of virus infection was defined as day 0 in the following experiments. All experiments were performed in accordance with the institutional guidelines of the Kyoto University.

2.3. Measurement of serum KP metabolites

For TRP, KYN, kynurenic acid (KA), and anthranilic acid (AA) measurements, serum was diluted (4:1, v/v) in 10% perchloric acid. After thorough mixing, the precipitated proteins were removed by centrifugation (7,000 × *g*, 10 min, 4 °C). Fifty microliters of the resulting supernatant was subjected to high-performance liquid chromatography (HPLC; SHIMADU) analysis. TRP, KYN, KA, and AA were isocratically eluted from a reverse phase column (TSKgel ODS–100 V, 3 μm, 4.6 mm (ID) × 150 mm (L)) (Tosoh, Tokyo, Japan) using a mobile phase containing 10 mM sodium acetate and 1% acetonitrile (pH adjusted to 4.5 with acetic acid) at a flow rate of 0.9 ml/min. TRP and KYN were detected using an ultraviolet and visible spectrophotometric apparatus (SPD-20A, Shimadzu, Kyoto, Japan) (UV wavelength for Trp: 280 nm, UV wavelength for KYN: 365 nm). AA and KA were detected by a fluorescence detector (RF-20Axs) (Shimadzu, Kyoto, Japan) under the following conditions: for AA, the excitation wavelength was 320 nm, and the emission wavelength was 420 nm; for KA, the excitation wavelength was 334 nm and the emission wavelength was 380 nm.

For 3-HK measurement, serum was diluted (1:4, v/v) in 10% perchloric acid. After thorough mixing, the precipitated proteins were removed by centrifugation (7,000 × *g*, 10 min, 4 °C). Twenty microliters of the supernatant were applied to a 3-μm HPLC column (HR-80; 80 mm × 4.6 mm) (ESA, Chelmsford, MA), using a mobile phase consisting of 1.5% acetonitrile, 0.9% triethylamine, 0.59% phosphoric acid, 0.27 mM EDTA, and 8.9 mM sodium heptane sulfonic acid, at a flow rate of 0.5 ml/min. 3-HK was detected electrochemically using an ECD 300 detector (oxidation potential: +0.55 V) (Eicom, Kyoto, Japan) as described previously [17]

2.4. RNA extraction and real-time PCR analysis

Total RNA was extracted from cardiac tissue using Isogen (Nippon Gene, Tokyo, Japan), and RNA concentration was determined spectrophotometrically at 260 nm. RT-PCR was performed by using Revetra Ace Kits (Toyobo, Osaka, Japan).

The following PCR primers were used: EMCV, sense, 5′-GTCGTGAAGGAAGCAGTTCC-3′, and antisense, 5′-CACGTGGCTTTTGGCCGCAGAGGC-3′. KMO, sense, 5′-GTTATTGGCGGTGGTTTGGTTG-3′, and antisense, 5′-GGGCCAAGTTAATGCTCCTTC-3′. 18s, sense, 5′-GGATTGACAGATTGATAGC-3′, and antisense, 5′-TATCGGAATTAACCAGACAA-3′. EMCV, KMO, and 18 s were quantified by using real-time PCR in a 7900HT Fast Real-Time system (Applied Biosystems, Foster City, CA). The EMCV, KMO, and 18 s reactions were performed using Ssoadvanced SYBR Green Supermix (Bio Rad, Hercules, CA). The data were analyzed with the 7900HT software (version 2.3; Applied Biosystems). Heart homogenate from EMCV-infected WT mice on day 2 was used as a standard for expression of EMCV. Liver homogenate from WT mice was used as a standard for expression of KMO and 18s. The expression values of EMCV, KMO and 18 s were interpolated from the standard curve. RT-PCR data were calculated as the ratio of gene to 18 s expression by the relative quantification method. Data of KMO mRNA are presented as normalized transcript expression in the samples relative to normalized transcript expression in control mice (uninfected).

2.5. Measurement of serum cytokines and chemokines

The cytokines and chemokines in the serum samples were measured using a 23-plex kit, which is a multiplex detection kit (Bio-Rad, Hercules, CA). This kit detects the levels of CCL2, CCL3, CCL4, CCL5, CXCL1, TNF-α, IL-1β, IL-6, IL-10, IL-1α, IL-2, IL-3, IL-4, IL-5, IL-9, IL-12(p40), IL-12(p70), IL-13, IL-17A, G-CSF, GM-CSF, IFN-γ, and Eotaxin. The assay was performed according to the manufacturer's protocol. Fifty microliters of each prepared sample was diluted at a ratio of 1:3 in the sample diluent. All samples were