Life Sciences 118 (2014) 288–296

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Endothelin receptor antagonist exacerbates autoimmune myocarditis in mice

Kazuko Tajiri *, Satoshi Sakai, Taizo Kimura, Tomoko Machino-Ohtsuka, Nobuyuki Murakoshi, Dongzhu Xu, Zheng Wang, Akira Sato, Takashi Miyauchi, Kazutaka Aonuma

Cardiovascular Division, Faculty of Medicine, University of Tsukuba, Tsukuba, Ibaraki, Japan

ARTICLE INFO

Article history:
Received 15 October 2013
Accepted 8 January 2014
Available online 18 January 2014

Keywords:
Autoimmune myocarditis
Endothelin
Suppressor of cytokine signaling
Inflammation

ABSTRACT

Aims: Myocarditis and subsequent dilated cardiomyopathy are major causes of heart failure in young adults. Experimental autoimmune myocarditis (EAM) is a mouse model of post-infectious myocarditis and inflammatory cardiomyopathy. The pathological role of endothelin (ET) in myocarditis has not been elucidated.

Main methods: EAM was induced by immunization of cardiac myosin peptide with complete Freund's adjuvant on days 0 and 7 in BALB/c mice. An ET_A/ET_B dual receptor antagonist, SB209670, was administered by a continuous infusion from a subcutaneous pump for 2 weeks.

Key findings: An increase in the heart-to-body weight ratio was observed in SB209670-treated mice compared with vehicle-treated mice. Heart pathology in SB209670-treated mice was remarkable for gross inflammatory infiltration, in contrast to the lesser inflammation in the hearts of vehicle-treated mice. We found that an ET blockade decreased the number of $Foxp3^+$ regulatory T cells in the heart. The ET blockade also inhibited the expression of the suppressor of cytokine signaling 3 that plays a key role in the negative regulation of both Toll-like receptor- and cytokine receptor-mediated signaling. EAM is a $CD4^+$ T cell-mediated disease. $CD4^+$ T cells isolated from SB209670-treated EAM mice produced less IL-10 and more inflammatory cytokines, IL-6 and IL-17, than those isolated from vehicle-treated mice.

Significance: The ET receptor antagonist exacerbated autoimmune myocarditis in mice. Our novel findings suggest that ET may play an important role in the regulation of inflammation in myocarditis.

Introduction

Myocarditis is an inflammatory disease of the myocardium and one of the leading causes of dilated cardiomyopathy (DCM), which is currently the most frequent reason for heart transplantation (Kindermann et al., 2012). Viral infections are thought to be the most common primary cause of myocarditis in Western countries (Leuschner et al., 2009). Although its pathogenesis remains unclear, there is substantial evidence that a post-viral autoimmune-mediated response to cardiac antigens critically contributes to the development and progression of myocarditis (Kindermann et al., 2012; Lazzerini et al., 2013). On this basis, two animal models of experimental myocarditis have been used and have greatly advanced our knowledge of the pathogenesis of the disease; virus-induced myocarditis and experimental autoimmune myocarditis (EAM) (Lazzerini et al., 2013).

Endothelin (ET) has emerged as an important participant in the pathophysiology of a variety of cardiovascular diseases (Barton et al., 1998; Murakoshi et al., 2002; Sakai et al., 1996; Stewart et al., 1991). Experimental and clinical studies have demonstrated that the activation of the ET system in many diseases is characterized by inflammation or fibrotic remodeling. Several ET antagonists have been discovered, which has helped elucidate the mechanisms by which ET mediates its effects. There are a few publications on the effects of ET receptor blockers on virus-induced myocarditis models (Marchant et al., 2009; Ono et al., 1999; Seta et al., 2000), and the efficacy of an ET receptor blockade on viral myocarditis is still controversial. In a mouse model of Coxsackievirus B3 (CVB3)-induced myocarditis, Bosentan, an ET_A/ET_B dual receptor blocker, improved cardiac function, but enhanced the viral load and myocarditis severity through ET_A receptor mediated p38 MAPK activation (Marchant et al., 2009). Thus, antagonism of ET signaling is potentially a desirable therapeutic strategy for cardiac dysfunction; however it is still unclear whether an ET blockade is useful for controlling the inflammation itself or not.

To answer this question, we evaluated the effect of an ET receptor blockade on EAM in mice. EAM was induced by immunization with cardiac myosin peptide, which allowed us to examine the effect of ET receptor blockers on myocardial inflammation under infectious pathogen-free conditions.

* Corresponding author at: Cardiovascular Division, Faculty of Medicine, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8575, Japan. Tel./fax: +81 29 853 3143.
E-mail address: ktajiri@md.tsukuba.ac.jp (K. Tajiri).

http://dx.doi.org/10.1016/j.lfs.2014.01.007

Materials and methods

Study approval

All animal experiments were approved by the Institutional Animal Experiment Committee of the University of Tsukuba.

Mice

BALB/c mice were purchased from CLEA Japan. We used 6- to 8-week-old male mice.

Immunization protocol

The mice were immunized with 100 μg of murine cardiac α-myosin heavy chain (MyHC-α) peptide (MyHC-$\alpha_{614-629}$ [Ac-RSLKLMATLFSTYASADR-OH]; Toray Research Center) emulsified 1:1 in phosphate buffered saline (PBS)/complete Freund's adjuvant (CFA) (1 mg/ml; H37Ra; Sigma-Aldrich) on days 0 and 7 as described previously (Sonderegger et al., 2008; Tajiri et al., 2012, 2013).

Treatment

The mice were given either an ET_A/ET_B dual receptor antagonist, SB209670 [(+)-(1S,2R,3S)-3-(2-carboxymethoxy4-methoxyphenyl)-1-(3,4-methylenedioxyphenyl)-5-(prop-1-yloxy)indane-2-carboxylic acid] (n = 15) (10 mg/kg/day: SmithKline Beecham Pharmaceuticals), or saline (n = 15) (vehicle), subcutaneously using an ALZET® mini-osmotic pump (Model 2002, DURECT Corporation), implanted in the back. We previously investigated the effect of SB209670 on the development of hypertension, cardiac hypertrophy, glomerulosclerosis, and renal vascular wall thickening in transgenic hypertensive mice with over-expression of both human renin and angiotensinogen genes (Maki et al., 2004). The dose of the S209670 (10 mg/kg/day) was confirmed to be adequate to completely inhibit the effects of the ET-1 administered in the mice. The mice were treated for 2 weeks from day 0 to day 14 after immunization and sacrificed on day 14 for further analyses.

Histopathological examination

Myocarditis severity was scored on hematoxylin and eosin (H&E)-stained sections using grades from 0 to 4: 0, no inflammation; 1, less than 25% of the heart section involved; 2, 25 to 50%; 3, 50 to 75%; and 4, more than 75% as described previously (Sonderegger et al., 2008; Tajiri et al., 2012, 2013). Two independent researchers scored the slides separately in a blinded manner.

Flow cytometric analyses and intracellular cytokine staining

Heart inflammatory cells were isolated and processed as previously described (Eriksson et al., 2003; Valaperti et al., 2008). For the flow cytometric analysis of the surface markers and cytoplasmic cytokines, the cells were stained directly with conjugated fluorescence antibodies and analyzed with a FACSCalibur instrument (BD Biosciences). For the analysis of the intracellular cytokine production, the cells were stimulated with 50 ng/ml of phorbol 12–myristate 13–acetate (PMA), 750 ng/ml of ionomycin (Sigma-Aldrich) and 10 μg/ml of brefeldin A (eBioscience) for 5 h. The fluorochrome-conjugated, mouse-specific monoclonal antibodies purchased from eBioscience, included CD4 and forkhead box P3 (Foxp3).

Cytokine ELISA

For the analysis of the cytokines and chemokines in the heart, the hearts were homogenized in media containing 2.5% fetal bovine serum. The supernatants were collected after centrifugation and stored at −80 °C. The concentrations of cytokines and chemokines in the heart homogenates and culture supernatants were measured with Quantikine ELISA kits (R&D Systems).

Isolation of dendritic cells (DCs) and $CD4^+$ T cells

We used magnetic-activated cell sorting kits for the cell isolation (CD11c MicroBeads for DC isolation, $CD4^+CD62L^+$ T Cell Isolation Kit II for naïve $CD4^+$ T-cell isolation and $CD4^+$ T Cell Isolation Kit II for $CD4^+$ T-cell isolation, Miltenyi Biotec).

In vitro T-cell differentiation

Purified naïve $CD4^+CD62L^+$ T cells were stimulated with anti-CD3 1 μg/ml (R&D Systems) and anti-CD28 1 μg/ml (Acris Antibodies), with or without ET-1 under T helper (Th)1-, Th2-, Th17- or Treg polarizing conditions for 48 h. Th1 condition: IL-12 (10 ng/ml) and anti-IL-4 antibody (10 μg/ml). Th2 condition: IL-4 (10 ng/ml), anti-IL-12 (10 μg/ml), and anti-IFN-γ (10 μg/ml). Th17 condition: transforming growth factor (TGF)-β (10 ng/ml), IL-6 (20 ng/ml), IL-23 (20 ng/ml), anti-IL-4 (10 μg/ml), anti-IL-12 (10 μg/ml) and anti-IFN-γ (10 μg/ml). Treg condition: TGFβ1 (10 ng/ml), anti-IL-4 (10 μg/ml), anti-IL-12 (10 μg/ml), and anti-IFN-γ (10 μg/ml). The cytokines and antibodies were obtained from the R&D Systems except for the TGF-β (BioLegend).

Quantitative real-time reverse transcription polymerase chain reaction (QRT-PCR)

The total RNA was prepared using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The cDNA was synthesized from 1 μg of the total RNA by reverse transcriptase (Takara). QRT-PCR analysis was performed with LightCycler (Roche Diagnostics). The oligonucleotides used for the PCR amplification were the following: *Ece1* forward, TGGAGGTTATGTATGGGACGA; *Ece1* reverse, GGTTGTTTTCCG TGTCACTCA; *Edn1* forward, TCCTTGATGGACAAGGAGTGT; *Edn1* reverse, CCCAGTCCATACGGTACGA; *Edn2* forward, AGACCTCCTCCGAAAGCTG; *Edn2* reverse, TTTCTTGTCACCTCTGGCTGTA; *Edn3* forward, GCACCAGA GATGTCACCAGTT; *Edn3* reverse, AGTCTCCCGCATCTCTTCTG; *Ednra* forward, TGTGAGCAAGAAATTCAAAAATTG; *Ednra* reverse, ATGAGGCTTT TGGACTGGTG; *Ednrb* forward, TAGAGGCAACCGGGCTAGT; *Ednrb* reverse, GGGGAGTGAAGACAGGACAC; *Ifng* forward, ATCTGGAGGAACTG GCAAAA; *Ifng* reverse, TTCAAGACTTCAAAGAGTCTGAGGTA; *Il4* forward, CATCGGCATTTTGAACGAG; *Il4* reverse, CGAGCTCACTCTCTGTGGTG; *Il17a* forward, TGTGAAGGTCAACCTCAAAGTCT; *Il17a* reverse, GAGGGA TATCTATCAGGGTCTTCAT; *Socs3* forward, ATTTCGCTTCGGGACTAGC; *Socs3* reverse, AACTTGCTGTGGGTGACCAT; *Hprt* forward, TCCTCCTCAG ACCGCTTTT; and *Hprt* reverse CCTGGTTCATCATCGCTAATC. The data were normalized by the level of the *Hprt* expression in each sample.

Statistical analysis

Statistical analyses were performed using the two-tailed *t* test or Mann–Whitney *U* test, for experiments comparing two groups. For multiple comparisons, one-way analysis of variance with Dunnett's post-hoc test was used. *P* values < 0.05 were considered statistically significant.