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Original article

Activation of invariant natural killer T cells by α-galactosylceramide ameliorates myocardial ischemia/reperfusion injury in mice

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ABSTRACT

Invariant natural killer T (iNKT) cells orchestrate tissue inflammation via regulating various cytokine productions. However the role of iNKT cells has not been determined in myocardial ischemia/reperfusion (I/R) injury. The purpose of this study was to examine whether the activation of iNKT cells by α-galactosylceramide (α-GC), which specifically activates iNKT cells, could affect myocardial I/R injury. I/R or sham operation was performed in male C57BL/6J mice. I/R mice received the injection of either αGC (I/R + αGC, n = 48) or vehicle (I/R + vehicle, n = 49) 30 min before reperfusion. After 24 h, infarct size/area at risk was smaller in I/R + αGC than in I/R + vehicle (37.8 ± 2.7% vs. 47.1 ± 2.5%, $P < 0.05$), with no significant changes in area at risk. The numbers of infiltrating myeloperoxidase- and CD3-positive cells were lower in I/R + αGC. Apoptosis evaluated by TUNEL staining and caspase-3 protein was also attenuated in I/R + αGC. Myocardial gene expression of tumor necrosis factor-α and interleukin (IL)-1β in I/R + αGC was lower to 46% and 80% of that in I/R + vehicle, respectively, whereas IL-10, IL-4, and interferon (IFN)-γ were higher in I/R + αGC than I/R + vehicle by 2.0, 4.1, and 9.6 folds, respectively. The administration of anti-IL-10 receptor antibody into I/R + αGC abolished the protective effects of αGC on I/R injury (infarct size/area at risk: 53.1 ± 5.2% vs. 37.4 ± 3.5%, $P < 0.05$). In contrast, anti-IL-4 and anti-IFN-γ antibodies did not exert such effects. In conclusion, activated iNKT cells by αGC play a protective role against myocardial I/R injury through the enhanced expression of IL-10. Therapies designed to activate iNKT cells might be beneficial to protect the heart from I/R injury.

1. Introduction

Early and successful myocardial reperfusion is the most effective strategy to reduce infarct size and preserve cardiac function after acute myocardial infarction (MI) [1]. Reperfusion after ischemia can salvage the ischemic myocardium, however, simultaneously it causes additional cell death and attenuates the beneficial effects of reperfusion itself, called myocardial ischemia/reperfusion (I/R) injury [2]. Inflammation has been shown to play a critical role in the pathophysiology of myocardial I/R injury [3], and various immune cells, such as neutrophils, T lymphocytes, monocytes/macrophages, and mast cells, are involved in myocardial I/R injury [4–7]. Recent study by Yang et al. demonstrated that $CD4^+$ T lymphocytes played an important role in the development of I/R injury and interferon (IFN)-γ was involved in their action by using Rag1 knockout mice lacking mature lymphocytes [5].

Invariant natural killer T (iNKT or type 1 NKT) cells are innate-like T lymphocyte population characterized by co-expressing NK lineage receptors and T cell receptors (TCR), and their TCR has invariant α-chain (Vα14-Jα18 in mice, and Vα24-Jα18 in humans) [8,9]. They are activated by recognizing glycolipid antigens presented by CD1d, a member of major histocompatibility complex (MHC) class I

Abbreviations: AAR, area at risk; αGC, α-galactosylceramide; IFN-γ, interferon-γ; IL, interleukin; iNKT, invariant natural killer T; I/R, ischemia/reperfusion; IS, infarct size; LV, left ventricle; MI, myocardial infarction; MNCs, mononuclear cells; MPO, myeloperoxidase; NK, natural killer; qRT-PCR, quantitative reverse transcriptase-polymerase chain reaction; TCR, T cell receptor; T_H1, T-helper type 1; T_H2, T-helper type 2; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α; TTC, 2,3,5-triphenyltetrazolium chloride.

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like molecules, and rapidly secrete a mixture of large amount of T-helper type 1 (T_H1) and T_H2 cytokines, such as IFN-γ, interleukin (IL)-10 and IL-4 in shaping subsequent adaptive immune responses [10]. Thus, iNKT cells can function as a bridge between the innate and adaptive immune systems, and orchestrate tissue inflammation.

iNKT cells have been demonstrated to play a protective role in various autoimmune and inflammatory diseases such as type 1 diabetes, experimental allergic encephalomyelitis, rheumatoid arthritis, and enteritis [11–15]. We have also reported that the activation of iNKT cells by α-galactosylceramide (αGC), a specific activator for iNKT cells [16], can attenuate the development of left ventricular (LV) remodeling and failure after MI created by chronic ligation of coronary artery in mice [17]. The activation of iNKT cells by αGC has also been reported to protect the liver against I/R injury in mice via IL-13 production [18]. However, no previous studies have examined the effects of iNKT cell activation by αGC on myocardial I/R injury.

Therefore, the purpose of the present study was to determine whether the activation of iNKT cells by αGC could attenuate myocardial I/R injury. We also determined whether the protective effects on attenuated myocardial I/R injury might involve the activation of anti-inflammatory cytokines including IL-10.

2. Materials and methods

Detailed methods are available in the Online Supplementary Material.

2.1. Animals

C57BL/6J mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). Animals were used for experiments at 10 to 12 weeks of age (weight 23–27 g). Mice were bred in a pathogen-free environment and kept under a constant 12-h light–dark cycle at a temperature of 23 °C to 25 °C. Standard chaw and water were provided.

All procedures and animal care were approved by our institutional animal research committee and conformed to the animal care guideline for the Care and Use of Laboratory Animals in Hokkaido University Graduate School of Medicine.

2.2. Experimental design

2.2.1. Experiment 1: effects of αGC on iNKT cell and cytokine expression in the normal mice

To confirm that αGC could activate iNKT cells in the heart similar to the spleen, C57BL/6J mice were sacrificed 0, 24, and 72 h after αGC (Funakoshi Co., Ltd., Tokyo, Japan) injection (0.1 μg/g body weight i.p., n = 9 for each group) and the proportion of iNKT cells in the heart and spleen were measured by flow cytometric analysis [17].

To determine that αGC could induce the changes of cytokines in the blood and the heart within 24 h, another group of C57BL/6J mice were sacrificed 0, 0.5, 1, 3, 6, 12, and 24 h after single injection of αGC (n = 6 for each group). Serum levels of IL-10, IL-4, and IFN-γ were measured by ELISA and their gene expressions in the heart were measured by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). TCR in iNKT cells has invariant α-chain encoded by Vα14-Jα18 gene segment in mice, and $J\alpha18^{-/-}$ mice lack iNKT cells. To confirm whether the changes of cytokines by the injection of αGC were due to the activation of iNKT cells, iNKT cell-deficient ($J\alpha18^{-/-}$) mice were sacrificed after single injection of αGC and same measurements were performed. They were provided from Dr. M. Taniguchi (RIKEN, Yokohama, Japan) and backcrossed 10 times to C57BL/6J.

2.2.2. Experiment 2: effects of αGC on myocardial I/R injury

Myocardial I/R surgery or sham operation was performed in C57BL/6J mice according to the methods described previously [5]. After anesthesia, the left coronary artery was ligated for 45 min. Ischemia was confirmed by bleaching of the myocardium. Reperfusion was initiated by releasing the ligature. Sham-operated mice underwent a similar procedure without ligation. αGC (0.1 μg/g body weight i.p.) was administered 30 min before reperfusion to specifically activate iNKT cells. As control, the same volume of vehicle was administered into sham and I/R mice.

Mice were sacrificed 24 h after reperfusion (sham + vehicle, n = 22; sham + αGC, n = 22; I/R + vehicle, n = 49, I/R + αGC, n = 48). These mice were divided into groups for some measurements. Another groups of mice were sacrificed 72 h after reperfusion for flow cytometric analysis (n = 9 for each group), because iNKT cells have been reported to be invisible by flow-cytometric detection 24 h after αGC administration [19]. Additional mice were sacrificed 72 h after reperfusion for RT-PCR analysis (n = 7–8 for each group). To confirm early protective effect of αGC, C57BL/6J mice received I/R surgery with vehicle or αGC, and sacrificed 2 h after reperfusion to measure infarct size (I/R + vehicle, n = 7; I/R + αGC, n = 8). Furthermore, to confirm the effect of αGC-induced reduction of infarct size on long-term LV function and remodeling, echocardiography and hemodynamic measurement were performed at 28 days after reperfusion (I/R + vehicle, n = 8; I/R + αGC, n = 8).

To confirm whether the effect of αGC on infarct size in I/R was due to the activation of iNKT cells, $J\alpha18^{-/-}$ mice received I/R surgery with vehicle or αGC and sacrificed 24 h after reperfusion to measure infarct size (n = 5 for each group).

Furthermore, to examine the role of various cytokines in the effects of αGC on myocardial I/R injury, rat anti-IL-10 receptor monoclonal antibody (200 μg/mouse, i.p., BD Pharmingen, San Diego, CA), rat anti-IL-4 monoclonal antibody (250 μg/mouse, i.p., R&D System, Inc.), or rat anti-IFN-γ monoclonal antibody (150 μg/mouse, i.p., R&D System, Inc.) was administered 90 min before I/R surgery and infarct size was measured 24 h after reperfusion. The doses of these antibodies were chosen based on the previous study of their efficacy [18,19,20]. We also confirmed that the changes of serum IL-4 or IFN-γ levels were completely inhibited by identical antibodies. Rat IgG1κ was used as control. αGC was administered 30 min before reperfusion (I/R + αGC + rat IgG1κ, n = 8; I/R + αGC + anti-IL-10R, n = 8; I/R + αGC + anti-IL-4, n = 7; I/R + αGC + anti-IFN-γ, n = 9).

Finally, to examine the role of IFN-γ on myocardial I/R injury, rat anti-IFN-γ monoclonal antibody (150 μg/mouse, i.p., R&D System, Inc.) or IgG1κ was administered 90 min before I/R surgery and infarct size was measured 24 h after reperfusion (n = 6 for each).

2.3. Statistical analysis

Data are expressed as means ± SE. The Student *t* test was performed for comparison between 2 independent groups. For multiple-group comparisons, one-way ANOVA followed by the Dunnett's test or the Tukey's test was performed. A value of $P < 0.05$ was considered statistically significant.

The authors had full access to and take full responsibility for the integrity of the data. All authors had read and agree to the manuscript as written.

3. Results

3.1. Experiment 1: effects of αGC on iNKT cell and cytokine expression in the normal mice

3.1.1. Proportion of iNKT cells after αGC administration

After αGC administration, splenic iNKT cells disappeared at 24 h and were increased at 72 h (Supplemental Fig. 1, upper panel) in consistency with the previous report [19]. The number of cardiac iNKT cells itself was lower than that of splenic iNKT cells. However, they were increased 72 h after αGC administration in parallel to splenic