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Original article

Attenuation of experimental autoimmune myocarditis by blocking T cell activation through 4-1BB pathway

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ABSTRACT

4-1BB, a member of the tumor necrosis factor receptor (TNFR) family, binds the 4-1BB ligand (4-1BBL), works as a costimulatory molecule, and regulates T cell-mediated immune responses. Although inflammation is an essential pathological feature of myocarditis, the role of 4-1BB in experimental autoimmune myocarditis (EAM) remains unclear. Lewis rats were immunized on day 0 with purified porcine cardiac myosin to establish EAM. 4-1BB-immunoglobulin (4-1BBIg) was administered intraperitoneally ($n=6$) a total of 9 times (3 times per week). Rats were killed on day 21 to study effects of 4-1BB/4-1BBL pathway blockade. For controls, isotype-matched human IgG was administered in other EAM rats ($n=6$). Histologic and echocardiographic examination showed development of EAM attenuated by 4-1BBIg. Suppression of mRNA expression for IL-1α, IL-1β, IL-4, IL-6, and TNF-α was noted in the heart tissue treated with 4-1BBIg. Treatment with 4-1BBIg reduced production of Th1-type cytokines, and inhibited T cell proliferation in vitro. In the 4-1BB signaling pathway in splenocytes, 4-1BBIg suppressed JNK, p38, and IκB activity but not that of ERK1/2. Blockade of T cell activation through the 4-1BB/4-1BBL pathway regulates development of EAM; therefore, 4-1BB may be an effective target for treating myocarditis.

1. Introduction

Acute myocarditis is a fatal disease and a major cause of dilated cardiomyopathy; however, the etiology of myocarditis is unclear, and an effective treatment does not yet exist [1]. Autoimmunity is important in myocarditis: in particular, a reaction to cardiac myosin following viral infection may contribute to development of myocarditis [2]. Experimental autoimmune myocarditis (EAM) is characterized by severe myocardial damage that includes the infiltration of mononuclear cells into the myocardium and the appearance of multinucleated giant cells [3]. This cellular infiltrate consists predominantly of T lymphocytes, monocytes, and macrophages, all of which are recruited from the circulation. EAM is used as an animal model of human giant cell myocarditis [4]. In fact, giant cell myocarditis may be caused by autoimmune mechanisms. It is distinguished from lymphocytic myocarditis, which is mainly induced by viral infection. EAM is induced by T cell activation [5].

Optimal activation of T lymphocytes requires two signals, one from processed antigen through T cell receptors and the other from costimulatory signal through costimulatory molecules. Only when the two receptors are engaged are T cells activated for clonal expansion through activation of NFB (nuclear factor-κB) and production of IL-2 and other inflammatory molecules. Through this mechanism, chronic inflammation such as myocarditis is initiated and maintained. Multiple costimulatory pathways are reported including the 4-1BB system, and the functions of these signals are differentially regulated depending on the disease and situation. Stimulation and/or blockade of these molecules show promise as therapeutic applications for control of pathological situations, including cancer, infection, transplantation, autoimmunity, and vascular diseases [6].

4-1BB is a member of the tumor necrosis factor receptor (TNFR) family and is expressed primarily on activated $CD4^+$ and $CD8^+$ T cells, activated NK (natural killer) cells, and activated NK T-cells. Recently, it was reported that 4-1BB is expressed on monocytes, neutrophils, and dendritic cells. 4-1BB ligand (4-1BBL) is expressed on antigen-presenting cells such as activated B cells, activated macrophages, and mature dendritic cells, and on a variety of cells such as cardiac myocytes and neurons [6]. The 4-1BB pathway can provide critical costimulatory signals for T cells independently of CD28 [7]. Recent studies showed that signaling through the 4-1BB pathway is important in various animal models. For example, an agonistic 4-1BB monoclonal antibody inhibits murine rheumatoid arthritis and systemic lupus erythematosus [8,9]. The 4-1BB pathway is also a promising target for cancer therapy [10]. In vitro studies showed that the 4-1BB pathway is involved in T cell proliferation, cytokine production, and stimulation of

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NF-κB activation [11]. However, the role of MAPKs (mitogen-activated protein kinases) at the signaling pathways of 4-1BB stimuli has not been clearly shown, especially in EAM. Administration of anti-4-1BBL antibody was shown to modestly prolong allograft survival in a murine cardiac transplantation model [12], and we also found that blockade of the 4-1BB pathway with 4-1BBIg attenuates graft arterial disease in cardiac allografts in a mouse model [13]. On the other hand, in the murine acute viral myocarditis model using Coxsackievirus B3, it was reported that inhibition of the 4-1BB pathway reduces cardiac damage [14,15]. However, there are no reports regarding the role of this molecule in the EAM model. Also, signaling pathways associated with 4-1BB inhibition have not been investigated.

In the present study, we investigated the effect of 4-1BB/4-1BBL pathway blockade in a rat model of EAM and investigated the signaling pathways required for 4-1BB, including ERK1/2 (extracellular signal-regulated kinase), JNK (stress-activated c-Jun N-terminal kinase), p38 kinase, and NFκB.

2. Methods

2.1. Animals

Male Lewis rats (7 weeks old; body weights 180 to 200 g) were purchased from CLEA Japan, Inc. (Tokyo, Japan). They were fed a standard diet and water and were maintained in compliance with the animal welfare guidelines of the Institute of Experimental Animals, Tokyo Medical and Dental University. Our guidelines are equivalent to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

2.2. Induction of myocarditis

Purified porcine cardiac myosin (Sigma Chemical Co., St Louis, MO, USA) was dissolved in 0.01 M phosphate-buffered saline (PBS) and emulsified with an equal volume of complete Freund's adjuvant (Difco, Lawrence, KS, USA) supplemented with *Mycobacterium tuberculosis* H37RA (Difco) at a concentration of 10 mg/ml. On day 0, rats were injected subcutaneously in the footpads with 0.2 ml of this emulsion, yielding an immunizing dose of 1.0 mg/body of cardiac myosin per rat [16,17].

2.3. Reagents

Anti-4-1BB antibody was purchased from Abcam plc (Cambridge, UK). Anti-4-1BBL antibody (clone number; M-17) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). FITC-conjugated anti-CD4 antibody, FITC-conjugated anti-CD8 antibody, FITC-conjugated anti-CD11b/c antibody, and streptavidin–PE were purchased from PharMingen (San Diego, CA, USA). Isotype-matched human IgG was obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). FITC-conjugated anti-BrdU (5-bromo-2′-deoxyuridine) monoclonal antibody was purchased from Chemicon International, Inc. (Temecula, CA, USA). Phospho-ERK1/2 (p-ERK1/2) antibody, ERK1/2 antibody, phospho-JNK (p-JNK) antibody, JNK antibody, phospho-p38 (p-p38) antibody, p38 antibody, phospho-IκB (p-IκB) antibody, and IκB antibody were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

2.4. Preparation of 4-1BBIg

The 4-1BBIg was prepared by constructing an adenovirus vector encoding an extracellular domain of human 4-1BB and Fc portion of human IgG. The cDNA encoding the extracellular domain of 4-1BB was amplified by reverse transcription-polymerase chain reaction (RT-PCR) with mRNA isolated from human peripheral blood leukocytes stimulated by mixed lymphocyte reaction as template. The PCR primers were 5′-TGTGAATTCTTCATCATGGGAAACAGCTGT-3′ and 5′-CTCAGATCTTGCGGAGAGTGTCCTGGCTC-3′.

The cDNA was inserted into the EcoRI and BamHI sites of a plasmid carrying IgG1-Fc DNA [18], and the sequence was verified. 4-1BBIg DNA was then removed by EcoRI/BamHI digestion, and the blunt-ended fragment was ligated into the SwaI site of pAxCAwt cosmid vector (Takara, Kyoto, Japan) to prepare recombinant adenovirus Ax4-1BBIg. Preparation of recombinant adenovirus was done with an Adenovirus Expression Vector Kit (Takara) according to the manufacturer's instructions. 4-1BBIg protein was purified from supernatant of Ax4-1BBIg-infected COS7 cells as described previously [19].

2.5. Treatment

The 4-1BBIg treatment group ($n=6$) was given 1 mg/kg 4-1BBIg intraperitoneally on days 0, 2, 4, 7, 9, 11, 14, 16, and 18. EAM group rats ($n=6$) received no treatment after immunization. As a control Ig group, rats ($n=6$) were treated with isotype-matched human IgG after immunization on the same schedule as the 4-1BBIg treatment group. Native rats were prepared as a normal control group ($n=6$) for hemodynamic and echocardiographic study and for histologic examinations. All rats were killed on day 21 to examine the effects of 4-1BB/4-1BBL pathway blockade.

2.6. Immunohistochemistry

Frozen sections (5 μm) were fixed in acetone for 10 min at 4 °C. To reduce nonspecific binding, sections were incubated with 10% normal rabbit serum at room temperature. To stain for CD4, CD8, and macrophages, sections were incubated with antibodies against CD4, CD8 (PharMingen), and CD68 (clone number; ED1, AbD Serotec, Oxford, UK), respectively, overnight at 4 °C. Sections were washed with PBS and then incubated with biotinylated secondary antibodies at room temperature for 30 min. Antigen-antibody conjugates were detected with avidin–biotin–horseradish peroxidase complex (Nichirei, Tokyo, Japan) according to the manufacturer's instructions. The complex was visualized with 3-amino-9-ethylcarbazole chromogen. Sections were counterstained with hematoxylin, and cell numbers were determined by counting stained lymphocytes in 20 high-power fields (HPFs). Cell counting was performed 3 times each by two independent investigators, and results were averaged.

2.7. FACS analysis

Myocardial infiltrating cells were isolated from rats with myocarditis on day 21 after immunization with myosin emulsion. Cardiac tissue was minced with a sterile razor blade and placed in 2.5%FCS HANKs solution (Sigma). After washing out blood cells, the minced heart was perfused with 4 mg/ml collagenase solution (Type 2, Worthington Biochemical Co., Lakewood, NJ, USA). This mixture was rocked at 37 °C for 2 to 3 h and then strained through a 70-mm nylon cell strainer (Becton Dickinson, Franklin Lakes, NJ, USA). Dead lymphocytes and red blood cells were removed by centrifugation for 10 min at 2000 rpm. Infiltrating cell fraction was isolated by the Percoll (Amersham Biosciences, Buckinghamshire, UK) density method. The middle layer was rich in infiltrating cells, whereas the myocyte-rich pellet was in the bottom layer. We carefully collected myocardial infiltrating cells from middle layer with a Pasteur pipette. Cells were incubated on ice with anti-4-1BB antibody and anti-4-1BBL antibody or isotype-matched control IgG. After incubation with biotinylated anti-rabbit or anti-goat IgG, cells were incubated on ice with FITC-conjugated anti-rat CD4, anti-rat CD8 antibody, or anti-rat CD11b/c antibody and streptavidin–PE. Cells were washed with PBS and incubated with propidium iodide. Cells were then analyzed by flow cytometry with a FACScalibur system (Becton Dickinson) and CellQuest software.