Expression of Costimulatory Molecule CD40 in Murine Heart With Acute Myocarditis and Reduction of Inflammation by Treatment With Anti-CD40L/B7-1 Monoclonal Antibodies

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Abstract—Evidence has accumulated that antigen-specific T cells infiltrate the heart and play an important role in the pathogenesis of viral myocarditis. Costimulatory molecules such as B7s and CD40 expressed on antigen-presenting cells are known to play a critical role for antigen-specific T-cell activation to occur. To investigate the role for a costimulatory molecule, CD40, in the development of acute viral myocarditis, we first analyzed the expression of CD40 in the hearts of mice with acute viral myocarditis induced by Coxsackievirus B3. We also evaluated the induction of CD40 in cultured cardiac myocytes treated with interferon gamma in vitro. Second, we analyzed the cytokine production by cultured cardiac myocytes by stimulation with anti-CD40 monoclonal antibody (mAb) in vitro. Third, we examined the effects of in vivo administration of anti–CD40L/B7-1 mAbs on the development of acute viral myocarditis. We found that Coxsackievirus B3–induced murine acute myocarditis results in enhanced expression of CD40 on cardiac myocytes. The expression of CD40 on cardiac myocytes could be induced by interferon gamma in vitro. We also found that the production of interleukin-6 by cardiac myocytes was stimulated with anti-CD40 mAb and that in vivo anti–CD40L/B7-1 mAb treatment significantly decreased the myocardial inflammation. Our findings strongly suggest that CD40 plays an important role in the development of acute viral myocarditis and raise the possibility of immunotherapy with anti–CD40L/B7-1 mAbs to prevent T cell–mediated myocardial damage in viral myocarditis. (Circ Res. 1998;83:463-469.)

Key Words: myocarditis ■ cardiomyopathy ■ immunology ■ immunohistochemistry ■ polymerase chain reaction

A cell-mediated autoimmune mechanism has been strongly implicated in the pathogenesis of myocardial cell damage involved in viral myocarditis and dilated cardiomyopathy.1-7 We have previously reported that in murine acute viral myocarditis, natural killer (NK) cells infiltrate the heart first, then infiltration by antigen-specific T cells subsequently occurs, and major histocompatibility complex (MHC) class I antigen as well as intercellular adhesion molecule (ICAM)-1 are strongly induced on cardiac myocytes.2-4,8,9 This suggests that the expression of MHC class I antigen and ICAM-1 on cardiac myocytes facilitates the interaction between cardiac myocytes and T cells, especially cytotoxic T lymphocytes (CTLs), and leads to further myocardial cell damage, which may result in the development of dilated cardiomyopathy. This is supported by the finding that activated and antigen-specific T cells infiltrate the hearts of patients with dilated cardiomyopathy as well as acute myocarditis.10

It is necessary for T cells to receive 2 signals from the antigen-presenting cell (APC) for antigen-specific T-cell activation to occur. The first signal is provided by T-cell receptor engagement with the antigen/MHC complex, and the second signal, termed the costimulatory signal, is provided by costimulatory molecules on the APC.11 Among them, B7 family molecules B7-1 (B7, CD80)12,13 and B7-2 (B70, CD86),14,15 which are the ligands for CD28 and CTLA-4 on T cells, are the most extensively characterized and appear to be the most critical. Recently, we found that the expression of B7-1 and B7-2 is induced on cardiac myocytes of mice infected with Coxsackievirus B3 (CVB3) and that in vivo treatment with anti–B7-1 monoclonal antibody (mAb) significantly reduced myocardial inflammation.16 Another costimulatory molecule, CD40, a member of the tumor necrosis factor (TNF) receptor superfamily, is expressed on various APCs such as B cells, macrophages, and dendritic cells, and its ligand CD40L (gp39) is expressed on activated T cells. CD40-CD40L interaction is known to induce expression of B7 antigens and cytokine production by APCs as well as to initiate T cell–dependent antibody responses.17-19

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The purpose of the present study was to investigate in more detail the T cell–mediated autoimmune mechanism, especially the roles for CD40-CD40L interaction as well as B7-CD28 interaction in the development of acute viral myocarditis. For this purpose, first, we analyzed the expression of CD40 in the heart tissue of mice with acute myocarditis induced by CVB3. Second, to confirm the expression of CD40 on cardiac myocytes, we analyzed by immunofluorescence the induction of CD40 on cultured murine cardiac myocytes by treatment with interferon (IFN)-γ. Third, to investigate whether CD40-CD40L interaction induces cardiac myocytes to produce cytokines, we analyzed the cytokine production by cardiac myocytes stimulated with anti-CD40 mAb in vitro. Fourth, we examined the effects of in vivo treatment with anti-CD40L/B7-1 mAbs on the inflammation associated with acute viral myocarditis.

Materials and Methods

Virus and Animals

CVB3 (Nancy strain) was a kind gift from Dr Y. Kitaura (Osaka Medical College, Osaka, Japan). It was grown in cultures of FL cells (human amnion), which were supplied by the Japanese Cancer Research Bank–Cell Bank (National Institute of Hygienic Sciences, Tokyo). The virus preparation had a titer of $1 \times 10^7$ plaque-forming units (pfu)/mL and was stored at $-80°C$. Five-week-old C3H/He male mice and 14- to 16-day-old fetal C3H/He mice were purchased from Shizuoka Laboratory Animal Center (Shizuoka, Japan). Five-week-old mice were inoculated intraperitoneally with $1 \times 10^7$ pfu of CVB3 in 0.2 mL PBS.

Monoclonal Antibodies

An anti-mouse CD40L mAb (HM40L-1, hamster IgG) was purchased from PharMingen. The preparation of a mouse anti-cardiac myosin mAb (CMA19) has been previously described.1 The reactivity of CMA19 for C3H/He mouse ventricular myosin heavy chain was confirmed by immunoblot analysis (data not shown).

Preparation of Cultured Cardiac Myocytes

Primary cultures of ventricular cardiac myocytes were prepared from fetal C3H/He mice as previously described.2 The isolated ventricular myocytes were washed in DMEM containing 10% FCS, dispersed into plastic dishes for 1 hour to separate the fibroblasts, and then removed to LAB-TEK II chamber slides (Nalge Nunc International) for immunocytochemical study and to 24-well tissue culture plates (Iwaki Glass) for the study of cytokine production.

Immunohistochemistry

In the present study, to amplify the specific signals of antigen-antibody reaction, we used Tyramide Signal Amplification (TSA) technology for fluorescence (TSA-Direct [Green], NEN Life Science Products, according to the manufacturer’s instructions). Mice were killed on day 7 after virus inoculation. Cytostat sections (6-µm thick) of heart ventricles were prepared, air-dried, and fixed in acetone for 5 minutes at 4°C. After they were washed in PBS, the sections were incubated with hamster anti-mouse CD40 mAb for 1 hour at 37°C. After they were washed again in PBS, the sections were incubated with biotinylated goat anti-hamster IgG antibody (Caltag Laboratories Inc) for 1 hour at 37°C. The sections were then washed in TNT buffer (0.1 mol/L Tris-HCl, pH 7.5, 0.15 mol/L NaCl, and 0.05% Tween 20), blocked with TNB buffer containing a blocking reagent (0.1 mol/L Tris-HCl, pH 7.5, 0.15 mol/L NaCl, and 0.5% blocking reagent) for 30 minutes, and then incubated with streptavidin–hors eradish peroxidase for 30 minutes. After washing in TNT buffer, the sections were incubated with fluorescein-tetramethylrhodamine for the appropriate time (3 to 10 minutes), washed in TNT buffer, and then examined and photographed under a MICROPHOT-FX fluorescence microscope (Nikon).

Immunocytochemistry

For immunocytochemical analysis, to distinguish cardiac myocytes from nonmuscle cells (mainly consisting of fibroblasts), we performed double staining for cardiac myosin heavy chain and CD40. The isolated ventricular myocytes were divided into 2 groups, designated as A and B, and were cultured overnight at 37°C in a humidified 5% CO2/95% air incubator. After replacement with fresh culture media without FCS, recombinant murine IFN-γ (Shionogi & Co, Ltd) was added to group B to a concentration of $10^5$ U/L. After 48 hours under these conditions, the ventricular myocytes were subjected to immunocytochemical study. The cultured cells on the slides were washed in PBS and fixed in acetone for 5 minutes. They were incubated with CMA19 for 1 hour at 37°C, washed in PBS, incubated with TRITC-conjugated anti-mouse IgG antibody for 1 hour at 37°C, and washed again in PBS. The subsequent procedure for the staining of CD40 was the same as that for the tissue samples.

Measurement of Cytokines Produced by Cultured Ventricular Myocytes by Treatment With IFN-γ and Anti-CD40 mAb

The isolated ventricular myocytes ($3 \times 10^7$ cells/well) were divided into 4 groups, designated as A, B, C, and D, and were cultured overnight at 37°C in a humidified 5% CO2/95% air incubator. After replacement with fresh culture media without FCS (0.5 mL/well), recombinant murine IFN-γ was added to groups B and D to a concentration of $10^5$ U/L. After 24 hours under these conditions, anti-mouse CD40 mAb (HM40-3), which is known to stimulate CD40-expressing cells, was added to groups C and D to a concentration of 10 mg/L. Then, after 24 hours under these conditions, the culture supernatants were collected, and the concentration of interleukin (IL)-β, IL-6, IL-12 (p70), and TNF-α was measured using mouse ELISA kits for these cytokines (Endogen, Inc) according to the manufacturer’s instructions.

In Vivo Treatment of Mice With Anti-CD40L/B7-1 mAbs

Five-week-old C3H/He mice were divided into 4 groups, designated as A, B, C, and D (10 mice were used for each group). Mice in group B received the anti-CD40L/B7-1 mAb (5 mg/kg IP) on the day of virus inoculation (day 0) and on day 3. Mice in group C received the anti-B7-1 mAb (5 mg/kg), mice in group D received the anti-CD40L plus anti-B7-1 mAbs (5 mg/kg each), and mice in group A received hamster IgG (Organon Teknika Corp) for the control study, in the same way.

The hearts were removed and laterally sectioned approximately midway between the apex and the atria, which resulted in cross sections of both ventricles. Half of each heart was fixed in 10% buffered formalin and used for histological study.

Histology

The cross sections of formalin-fixed heart tissue from mice in each group were stained with hematoxylin and eosin and then photographed and printed onto color copy papers. The total area of the myocardium and the areas of inflammation (consisting of cell infiltration and necrosis) were accurately outlined on the color copy papers by microscopically examining the original hematoxylin/eosin-stained cross sections and scanned. The cross sections of formalin-fixed heart tissue from mice in each group were stained with hematoxylin and eosin and photographed. The percent area of the myocardium undergoing inflammation was determined by analysis performed on a Macintosh computer using the public domain NIH Image program (written by Wayne Rasband at the National Institute of Health).
Institutes of Health and available on the Internet by anonymous ftp from zippy.nimh.nih.gov or on floppy disk from NTIS, 5285 Port Royal Rd, Springfield, VA 22161, part number PB93-504868).

Statistical Analysis
For in vivo mAb-treatment study, one-way ANOVA (using $P$ values corrected by Bonferroni adjustment for multiple comparison) was used to evaluate differences between the groups. For the study of cytokine production by cultured ventricular myocytes, an unpaired $t$ test was used to evaluate differences between the groups. Values of $P < 0.05$ were considered significant.

Results
Expression of CD40 in Ventricular Tissue
In ventricular tissue of normal mice, CD40 was weakly to moderately expressed by many cells almost uniformly distributed over the myocardium (Figure 1A and 1C [higher magnification]). These cells positive for CD40 are thought to be dendritic cells. There was almost no expression of CD40 on cardiac myocytes of normal mice (Figure 1A and 1C). Also, there was almost no expression of CD40 on cardiac myocytes of mice on days 1 to 4 (data not shown). On day 5 after virus inoculation, just after massive cell infiltrations appeared, expression of CD40 was clearly induced on the sarcolemma of cardiac myocytes and the infiltrating cells and then reached a maximum level on about day 7 (Figure 1B and 1D [higher magnification]). The expression of CD40, which continued for $>4$ weeks after virus inoculation with gradual decrease, was seen nonuniformly over the myocardium and around the areas of cell infiltration. Ventricular myocardium infected with CVB3 (day 7) stained with nonimmune hamster sera as a negative control showed no significant level of signals (Figure 1E), indicating that nonspecific background was very low.

Induction of CD40 on Cultured Ventricular Myocytes by IFN-$\gamma$
Figure 2 shows double-stained ventricular myocytes cultured in a medium with or without IFN-$\gamma$ for 48 hours. Panels A and B show the staining pattern specific for CD40; panels C and D, which correspond to panels A and B, respectively, show the staining pattern specific for cardiac myosin heavy chain and indicate that most of the cells were cardiac myocytes. There was very slight or no expression of CD40 on the ventricular myocytes of the control group (panel A). After treatment with IFN-$\gamma$, most of the ventricular myocytes moderately to strongly expressed CD40 on their surfaces (panel B). No significant level of CD40 expression was induced on most of the nonmuscle cells, which mainly consisted of fibroblasts, by treatment with IFN-$\gamma$.

Figure 1. Immunohistochemical study for CD40 in ventricular tissue. Normal ventricular myocardium (A and C) and ventricular myocardium of mice on day 7 of CVB3 infection (B and D) were stained with anti-CD40 mAb. In normal ventricular myocardium, moderate expression of CD40 was observed on many dendritic cells, whereas almost no expression of CD40 was observed on sarcolemma of cardiac myocytes (A and C). In contrast, strong expression of CD40 was observed on the sarcolemma of many cardiac myocytes and the infiltrating cells in ventricular myocardium infected with CVB3 (day 7) (B and D). Ventricular myocardium infected with CVB3 (day 7) stained with nonimmune hamster sera as a negative control showed no significant level of signals (E). Bar=20 $\mu$m.

Figure 2. Immunocytochemical study of cultured ventricular myocytes for CD40. A and B, Myocytes in the control group (A) and in the IFN-$\gamma$-treated group (B) stained with anti-CD40 mAb and labeled with FITC by the TSA method. C and D, Myocytes stained with an antibody for cardiac myosin heavy chain (CMA19) and labeled with TRITC, corresponding to panels A and B, respectively. Most of the cells reacted in both groups, indicating that they were cardiac myocytes. Slight or no reactions to anti-CD40 mAb were seen in the control group (A), whereas strong reactions were seen on cardiac myocytes in the IFN-$\gamma$-treated group (B). Bar=20 $\mu$m.
IL-6 was significantly increased in the culture supernatants of cardiac myocytes in response to IFN-γ anti-CD40 mAb stimulation. As shown in Figure 3, undetectable levels or very low levels of IL-6 were produced by cardiac myocytes without treatment (control) or cardiac myocytes pretreated with IFN-γ only, respectively. In contrast, there was a significant and marked increase in the amount of IL-6 produced by cardiac myocytes stimulated with anti-CD40 mAb alone as well as with IFN-γ plus anti-CD40 mAb (both P < 0.001 versus control group and IFN-γ-stimulated group, respectively). There was a slight but significant difference in the amount of IL-6 between the anti-CD40 mAb-stimulated group and the IFN-γ plus anti-CD40 mAb-stimulated group. This suggested that induction of CD40 molecules on cardiac myocytes by IFN-γ could enhance CD40 signals into the cardiac myocytes, although the effect of IFN-γ itself on the IL-6 production by cardiac myocytes was minimal.

In Vivo Anti-CD40L/B7-1 mAb Treatment Study

The incidence of myocarditis was 100% in all of the groups. Panels A to D of Figure 4 show the representative sections of the heart of a mouse from group A (hamster IgG–treated control group), group B (anti-CD40L mAb–treated group), group C (anti–B7-1 mAb–treated group), and group D (anti-CD40L plus anti–B7-1 mAb–treated group), respectively. One mouse from group A died before being killed for study. Extensive cell infiltration and necrosis were seen in the mouse from group A (panel A), whereas both cell infiltration and necrosis were less severe in the mice from groups B, C, and D (panels B, C, and D, respectively). The results of the histological study are summarized in Figure 5. The (mean ± SD) percent area of myocardium undergoing inflammation was significantly decreased in group B (6.34 ± 3.24%, P < 0.05), group C (6.00 ± 2.67%, P < 0.05), and group D (4.39 ± 2.35%, P < 0.005) compared with group A (10.68 ±
of these antigens was clearly induced on cardiac myocytes on ing cells. This is also supported by the fact that the expression shown to be mainly synthesized by the infiltrating cells in cardiac myocytes in vitro, although there was some but not a marked difference only. The fact that anti-CD40 mAb treatment without IFN–CD40L plus anti–B7-1 mAb treatment and anti-CD40L interaction directly induces immune responses of APCs, such as cytokine production, and activates T cells through induction of B7 antigens on APCs or that the dose of anti-CD40L mAb administered may have been not sufficient to inhibit CD40-CD40L interaction. These data raise the possibility of immunotherapy with anti-CD40L mAb, with anti–B7-1 mAb, or with both to prevent myocardial damage in viral myocarditis.

It is known that CD4+ T-helper (Th) cells differentiate into 2 subsets capable of secreting distinct patterns of cytokines on antigenic stimulation. Th1 cells secrete IL-2, IFN-γ, and TNF-β, which activate macrophages and are critical for inducing cell-mediated immune responses. Th2 cells secrete IL-4, IL-5, and IL-10, which are critical for IgG1 and IgE antibody production and also suppress cell-mediated immune responses.

Roles for costimulatory molecules B7s/CTLA-4 and CD40/CD40L have been extensively studied in murine experimental autoimmune encephalomyelitis (EAE), one of the best characterized models of autoimmune diseases. Kuchroo et al have reported that administration of anti-B7-1 mAb results in predominant generation of Th2 clones and significantly suppresses the induction of this autoimmune disease, whereas administration of anti–B7-2 mAb facilitates Th1 development and substantially increases disease severity. Furthermore, Grewal et al have reported that CD40L-deficient mice fail to develop EAE and that these mice develop EAE when they receive B7-1+ APCs before being primed with antigen. This suggests that B7-1 expression on APCs, induced by CD40-CD40L interaction, is essential for the development of EAE and that treatment with anti-B7 mAbs can alter the course of autoimmune diseases by differentially influencing the development of Th1/Th2 cells from Th precursor cells. In vivo anti-CD40L mAb treatment has also been shown to prevent other autoimmune diseases, such as lupus nephritis and collagen-induced arthritis, as well as allograft rejection and graft-versus-host disease.

From the analysis of cytokine profile of the infiltrating cells, we found that this model of acute viral myocarditis is mainly mediated by Th1 cells like EAE. To investigate the effects of anti-CD40L plus anti–B7-1 mAb treatment on these immune mediators, we also examined the expression of Th cell–related cytokines, B7-1, and inducible NO synthase as well as CVB3 genomes in the heart tissues of mice by a semiquantitative polymerase chain reaction method. However, there was no significant difference in the expression of IFN-γ, IL-2, and IL-4 transcripts in the ventricular tissues between the anti-CD40L plus anti–B7-1 mAb–treated group and the hamster IgG–treated control group, suggesting that no
significant change in the ratio of infiltrating Th1/Th2 cells occurred (data not shown). There was also no significant difference in the expression of CVB3 genomes and inducible NO synthase transcript, which are thought to aggravate the inflammation\(^3\) (data not shown). However, we could not exclude the possibility that the polymerase chain reaction technique lacks sufficient resolution to discriminate the differences in the expression of these transcripts or that the lack of differences in the expression of transcripts may not reflect actual differences in the expression of these immune mediator proteins. We previously reported the strong expression of IFN-\(\gamma\) transcript by polymerase chain reaction in the heart tissue or by in situ hybridization in infiltrating cells in the heart tissue of this murine viral myocarditis.\(^3,4\) These data strongly suggested that the cardiac myocytes of mice with acute myocarditis were exposed to a high concentration of IFN-\(\gamma\) that was mainly synthesized by the infiltrating cells in vivo. Therefore, it seems that the expression of B7-1 and B7-2 could be induced on cardiac myocytes directly by IFN-\(\gamma\) in vivo even without CD40 signaling. We found by immunohistochemistry that there were similar levels of expression of B7-1 and B7-2 antigens on cardiac myocytes of mice in the control hamster IgG–treated group and those in the anti-CD40 mAb–treated group (data not shown).

It was shown that the expression of B7-1 on tumor cells induces an immune response mediated by CD8\(^+\) CTLs and leads to tumor regression.\(^5,6\) Therefore, it is thought that the expression of B7-1 on cardiac myocytes in acute viral myocarditis may facilitate infiltrating CTLs to directly injure cardiac myocytes and that IL-6 produced by the cardiac myocytes triggered by CD40 signaling may enhance the direct cytotoxicity of the CTLs against the cardiac myocytes. This may be one of the mechanisms of the in vivo effect of anti-CD40L/B7-1 mAb treatment. Recently, it has been reported that expression of B7-1 on tumor cells triggers NK cell–mediated cytotoxicity, which leads to tumor regression, and that this response occurs even in the absence of CD28 and cannot be inhibited by the expression of MHC class I antigen.\(^7,8\) This would also be one of the most likely explanations for the in vivo effect of anti-CD40L/B7-1 mAb treatment revealed in the present study, because NK cells consist of the dominant population of the infiltrating cells in the early stage of this murine viral myocarditis.\(^9\) IL-6 produced by the cardiac myocytes may also play an important role in the NK cell–mediated direct cytotoxicity against the cardiac myocytes.

Thus, at least CTLs and NK cells seemed to be involved in the mechanism of the in vivo effect of anti-CD40L/B7-1 mAb treatment. Further investigation is required to clarify whether the change in Th-cell differentiation was really induced and whether antigen-specific T-cell activation was suppressed by in vivo anti-CD40L/B7-1 mAb treatment.

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