An in vivo model of autoimmune post-coxsackievirus B3 myocarditis in severe combined immunodeficiency mouse

Chiharu Kishimoto a,*, Yuji Hiraoka a, Nami Takamatsu b, Hitoshi Takada a, Hajime Kamiya c, Hiroshi Ochiai b

a The Second Department of Internal Medicine, Faculty of Medicine, Toyama Medical and Pharmaceutical University, Japan
b Department of Human Science, Faculty of Medicine, Toyama Medical and Pharmaceutical University, Japan
c Department of Bioregulation, School of Medicine, Mie University, Japan

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Abstract

Objective: Severe combined immunodeficiency (SCID) mice possess neither T nor B lymphocytes and are thus suitable recipients for adoptively transferred lymphocytes. Because autoimmune mechanisms may be involved in the pathogenesis of coxsackievirus B3 (CB3) myocarditis, we attempted to assess the in vitro cellular damage caused by antigen-sensitized lymphocytes and to determine whether splenic lymphocytes from BALB/c mice with chronic CB3 myocarditis could cause myocarditis into SCID mice.

Methods and results: Cytotoxic cellular damage against non-myocytes and myocytes was demonstrated using 51Cr-release assay by lymphocytes cultured from myocarditis specimens or splenocytes from mice with chronic CB3 myocarditis. Severe T lymphocyte infiltration with myocardial necrosis was found in adoptively splenocyte-transferred SCID mice, but myocardial necrosis was not detected in wild-type mice by the same procedure.

Conclusions: Autoimmune mechanisms might operate in mice with post-CB3 myocarditis and could be transferred into SCID mice by the antigen-sensitized lymphocytes. An in vivo model of autoimmune post-viral myocarditis in SCID mouse was demonstrated.

Keywords: Myocarditis; Infection/inflammation; Immunology; Histology

1. Introduction

Viruses have often been implicated in the pathogenesis of autoimmune disorders in man, but proof of their etiologic role has been obtained in only a few diseases [1]. In human myocarditis, some evidence implicates virus-induced immunological mechanisms in the pathogenesis of the disease and in the persistent and progressive myocardial damage [2–4]. Strong evidence supports a role for cellular immune mechanisms in the pathogenesis of myocarditis and subsequent dilated cardiomyopathy [5–8]. In vitro studies of mononuclear cells from patients with myocarditis and dilated cardiomyopathy have been focusing on the characterization of cells in inflammatory infiltrates of heart muscle that has been shown to have T cells as active participants in myocardial damage.

Coxsackievirus B3 (CB3) is an enterovirus that can cause acute myocarditis in man [2,3]. We have shown previously that CB3 infection in various inbred strains of mice produces mild to severe myocarditis, which is followed by chronic myocardial dysfunction and congestive heart failure, and that the cells belonging to the Thy 1.2+ (pan T) and the Lyt 1+,2− (immature T) subsets are pathogenic in the development of myocarditis in mice [9–11]. Similar results were also demonstrated in murine myocarditis induced by encephalomyocarditis virus, a cardiotropic virus ordinarily not pathogenic to man [12,13]. However, the in vivo autoimmune murine model in post-CB3 myocarditis remains unestablished. If an in vivo animal model for the disease is established, the scientific merits might be of great value.
Severe combined immunodeficiency (SCID) mice lack both functional T and B lymphocytes because of a defective differentiation of both lymphocyte progenitors [14]. The SCID mutation maps to chromosome 16. The mice lack functional antigen receptors and thus allow allogenic immune system cells; it is possible to establish a functional murine immune system in the SCID mouse by the transfer of allogenic lymphocytes [15]. By the use of this approach, a variety of autoimmune diseases, such as systemic lupus erythematosus and rheumatoid arthritis, were transferred successfully [16,17]. These results indicate that the SCID mouse perfused with non-self lymphocytes serves as a valuable animal model for the evaluation of autoimmune diseases.

In this study, we investigate whether splenocytes from mice with chronic stage of CB3 myocarditis could transfer the disease into SCID mice. We create a new in vivo model to evaluate the autoimmune response in post-CB3 myocarditis.

2. Materials and methods

2.1. Virus and cells

Myocarditic CB3 (Nancy strain, American Type Culture Collection) was used. Virus stocks were prepared in cultures of VERO (kidney of African green monkey) cells in Eagle’s minimum essential medium (EMEM). Virus suspensions were centrifuged after the cytopathic effect had developed. Each virus stock had a titer of more than $10^9$ plaque-forming units (PFU) per 0.1 ml, determined by plaque assay. Virus was stored at $-80\,^\circ C$ until it was diluted for use.

Virus titers were determined by plaque formation on VERO cell monolayers as previously described [7–13].

2.2. Animals

Ordinary (wild-type) and SCID BALB/c(H-2d) mice 4–5 weeks old were used. They were maintained in filter-topped cages in a single, self-contained animal isolation room and handled with gloves by gowned and masked personnel. The intraperitoneal route was used for infection with virus.

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.3. Lymphocyte preparation

Spleens from control and virus-infected mice were removed aseptically. The lymphocytes were obtained by pressing the spleens through a fine mesh screen. After mincing, the cell suspension was pipetted rapidly with a sterile Pasteur pipette into 20–25 ml of Hanks’ balanced salt solution (HBSS), filtered through nylon mesh to eliminate debris, and centrifuged at 1500 rpm for 5 min. The cells were washed twice with HBSS. The lymphocyte fractions of these samples were obtained by Ficoll–Plaque (Pharmacia, Piscataway, NJ) gradient centrifugation; the suspension was layered carefully over 4 ml of Ficoll–Plaque and centrifuged at 1800 rpm for 15 min. The red blood cells were lysed by hypotonic shock. The lymphocytes were stained with 1.2% trypan blue and then counted in a standard hemocytometer. Cell viability was examined by trypan blue-dye exclusion test.

2.4. Lymphocyte culture

The precise method has been described previously [18]. In brief, BALB/c wild mice at 5–7 weeks of age (total $n=108$) were inoculated intraperitoneally with 0.1 ml of coxsackievirus B3 suspension containing 100–300 PFU. Mice were killed periodically (on days 21–35). After confirmation of myocarditis from the gross appearance of the heart (yellowish white patches on the surface), the heart was removed aseptically and cut into about 20 pieces (2–3 mm in length); one piece was processed for histologic study (hematoxylin–eosin stain), the others for lymphocyte culture. Lymphocytes were maintained in a 24-well flat-bottomed tissue culture plate by the replenishment of medium containing 10% of interleukin-2 (IL-2) with RPMI 1640 supplemented with 5–10% fetal calf serum (FCS) at $37\,^\circ C$ in a humidified atmosphere with 5% CO$_2$. Tissues were observed daily on an inverted microscope to monitor cells that exuded from the tissue. The medium was replenished at least every third day. Proliferating lymphocytes from positive wells were removed, pooled, and restimulated with phytohemagglutinin (PHA). When growth within a particular well was slow, medium was replenished by aspirating two-thirds of the medium and adding fresh medium to fill the well. After the confirmation of the absence of virus in cultured samples by plaque assay, lymphocytes were processed for transfer method.

2.5. Cytotoxic cellular damage assay

This protocol was conducted to measure cytotoxic activity of the cultured lymphocytes or splenocytes from mice with chronic myocarditis against EL-4 tumor cells or cultured fetal myocytes by $^{31}$Cr-release assay. The precise method was already reported [19,20]. In brief, EL-4 tumor cells, benzol(a)pyrene-induced lymphoma of C57BL/6 mice(H-2b), were used for assay. The cells were so called natural killer cell-resistant and maintained in RPMI-1640 medium supplemented with 10% FCS. Single cell suspensions of myocytes were prepared; mouse heart ventricles were removed from 14-day-old fetuses and dissociated into single isolated cells by trypsinization and dissociated into single EMEM with 10% FCS at a final cell density of $10^5$ cells per 2 ml dish.
Spleens were mechanically dissociated, and the cell suspension was collected. Erythrocytes were lysed, and macrophages were removed. The cells residing on the interface were collected, washed, and resuspended in RPMI 1640 medium with 10% FCS.

Target cells (1 x 10^6) were labeled with 3.7 MBq ^51^Cr for 60 min at 37 °C in 5% CO_2_. Labeled target cells were washed three times, and 1 x 10^4 cells/well were added to flat-bottomed 96-well microtiter plates (Corning) with effector cells at effector/target cell (E/T) ratios of 3.13:1, 6.25:1, 12.5:1, and 25:1 for EL-4 cells and at an E/T ratio of 12.5:1 and 50:1 for cultured myocytes. After 4 h of incubation at 37 °C, the supernatant was collected using the supernatant-collecting system (Skatron), and the amount of ^51^Cr released into the supernatant fluid was measured. The percentage of cytotoxicity was calculated using the formula

\[
\% \text{ cytotoxicity} = \frac{(E - S)}{(M - S)} \times 100
\]

where \(E\) is the counts per minute (cpm) released in the presence of effector cells, \(S\) is cpm released from target cells incubated alone in medium, and \(M\) is cpm released target cells incubated alone followed by 0.5N HCl treatment. All experimental data are the mean of duplicate samples. The parallel experiments of measuring cytotoxic activities of control (normal) lymphocytes or splenocytes were performed.

2.6. Adoptive transfer of spleen cells

Spleen cells were suspended in HBSS as described previously [11,18]. Mice in the following experiment received spleen cells by intraperitoneal injection. That is, wild-type and SCID mice received splenocytes adoptively. Mice were observed daily until 14–21 days after the transfer method, when the mice were sacrificed. After gross inspection, the hearts were sectioned transversely in the mid-portion and processed for immunohistologic, virologic, or pathologic studies.

2.7. Histology

The hearts were cut at several levels; one was fixed in 10% formalin solution, embedded in paraffin, sectioned serially at 5 μm, and stained with hematoxylin–eosin. The others were processed for immunohistology. Myocardial cell necrosis and cellular infiltration were scored blindly and independently on a scale of 1+ to 4+ in terms of severity. A 0 score indicated no or questionable lesions. A 1+ score described a limited focal distribution of myocardial lesions. A 4+ score described the presence of multiple lesions over the entire heart, while scores of 2+ and 3+ were used to describe intermediate severity. The mean value was cited. Surface markers of myocardial infiltrating lymphocyte subsets were immunohistologically stained by indirect method, as previously described[10–13]. The positive controls were prepared to avoid the technical errors.

2.8. Virus detection of tissues

For infectivity assays, aliquots of hearts or spleens were removed, weighed, and homogenized in 2 ml of EMEM aseptically. After centrifugation at 1500 rpm for 15 min at 4 °C, supernatants were inoculated into VERO cell monolayers, and plaque assays were performed. In addition, polymerase chain reaction (PCR) for coxsackieviral RNA was performed to exclude the presence of

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Cytotoxic cell activity</th>
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</thead>
<tbody>
<tr>
<td>Groups</td>
<td>( n )</td>
</tr>
<tr>
<td></td>
<td>E/T ratio</td>
</tr>
<tr>
<td>Cultured lymphocytes from myocarditis specimens</td>
<td>4\textsuperscript{a}</td>
</tr>
<tr>
<td>Control lymphocytes\textsuperscript{a}</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>E/T ratio</td>
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<tr>
<td>Against EL-4 cells (mean ± S.D., %)</td>
<td></td>
</tr>
<tr>
<td>Splenocytes from myocarditis mice</td>
<td>8</td>
</tr>
<tr>
<td>Control splenocytes</td>
<td>8</td>
</tr>
</tbody>
</table>

\*p < 0.05 vs. controls.

\**p < 0.01 vs. controls.

\textsuperscript{a} Control lymphocytes were obtained from lymph nodes of normal mice and cultured in RPMI 1640 for 3 days before transfer method.

\textsuperscript{a} Duration of culture periods of samples was 98, 112, 118, and 162 days post-viral inoculation, respectively.
virus genome. In brief, RNA was isolated and treated with DNAse I. Primers were designed corresponding to the 5' untranslated region of the coxsackieviral RNA as previously described [21]. Nested reverse transcriptase-PCR was performed.

2.9. Statistics

Experimental data were examined by one-way analysis of variance with multiple-sample comparison (ANOVA). If the F test was significant, the Bonferroni test was used to

Table 2

<table>
<thead>
<tr>
<th>Groups</th>
<th>Post-viral inoculation (days)</th>
<th>Doses (×10^6)</th>
<th>n</th>
<th>Diseased/total</th>
<th>Microscopic scores (mean)</th>
<th>Immunopathology*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Splenocytes from myocarditis mice</td>
<td>SCID 21</td>
<td>2 × 10^7</td>
<td>9</td>
<td>6/9</td>
<td>1.5</td>
<td>2+</td>
</tr>
<tr>
<td></td>
<td>wild 21</td>
<td>2 × 10^7</td>
<td>6</td>
<td>0/6</td>
<td>0</td>
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<td>SCID 23</td>
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<td>10</td>
<td>8/10</td>
<td>2.1</td>
<td>0</td>
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<td>6</td>
<td>0/6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>SCID 25</td>
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<td>8</td>
<td>7/8</td>
<td>2.1</td>
<td>2+</td>
</tr>
<tr>
<td></td>
<td>wild 25</td>
<td>5 × 10^7</td>
<td>6</td>
<td>0/6</td>
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<td>0</td>
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<tr>
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<td>6</td>
<td>3/6</td>
<td>1.3</td>
<td>0</td>
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<tr>
<td></td>
<td>wild 35</td>
<td>5 × 10^7</td>
<td>6</td>
<td>0/6</td>
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<td>Control splenocytes</td>
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<td>6</td>
<td>0/6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>wild 5</td>
<td>5 × 10^7</td>
<td>6</td>
<td>0/6</td>
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</table>

*The scores of immunostaining were graded semiquantitatively on a scale of 1+ to 4+. Score 0 indicated the complete absence of staining; score 1+, positively stained associated with < 5% of the infiltrating cells; score 2+, positively with < 10% of the cells; score 3+, positively with < 30% of the cells; 4+, positively with ≥ 30% of the cells.
evaluate differences between subsets. A two-tailed $p < 0.05$ was considered a significant difference.

3. Results

3.1. Lymphocytes were cultured from the hearts of CB3 myocarditis

As previously demonstrated [18], lymphocytes grew out from myocarditis specimens in IL-2-containing medium in vitro. Briefly, many macrophages and fibroblasts first appeared in wells and thereafter gradually decreased. Lymphocytes migrated out of the tissue approximately 21–31 days and increased in number. Without the tissue, lymphocytes stopped to grow; that is, the continued presence of the specimens is necessary for the viability and growth of the lymphocytes. In some cases, the wells were exchanged from a 24-well type to a 6-well type keeping the same tissues.

3.2. Lymphocytes cultured from myocarditis specimens or splenocytes from myocarditis mice caused myocardial damage in vitro

Cytotoxic cellular damage was examined in vitro by cultured lymphocytes from myocarditis specimens or by splenocytes from mice with chronic myocarditis. As a result, cytotoxic cell activities against EL-4 cells and against myocytes by cultured lymphocytes from myocarditis specimens were significantly higher than those by control cultured lymphocytes. In addition, enhanced cytotoxic activities were demonstrated in splenocytes from mice with chronic myocarditis compared with control culture lymphocytes (Table 1).

3.3. Antigen-sensitized lymphocytes caused myocarditis in SCID mice, but not in wild mice

In the vivo experiment, we attempted to determine whether splenic lymphocytes from wild mice with aviremic, chronic CB3 myocarditis could cause myocardial injury in SCID mice. Myocarditis was detected in SCID mice by the transfer of splenocytes, but not in wild mice. The lesions were predominant around the epicardium of the ventricular walls. The presence of pan T and CD4 cells was documented in the myocardium of SCID mice (Fig. 1, Table 2).

3.4. Virus detection

To examine whether the recurrence of myocardial lesions was due to the presence of viruses in adoptively transferred spleen cells, we examined splenic and myocardial virus by the conventional biological method and PCR before the reconstitution study. As a result, no viruses from the spleens or hearts were recovered in the experiments (Fig. 2).

4. Discussion

In the present experiments, we demonstrated that it is possible to transfer the autoimmune phenomenon present in murine post-CB3 myocarditis into SCID mice but not into wild mice, by the antigen-sensitized lymphocytes, which were obtained from diseased mice at the chronic stage of myocarditis. A pathogenic significance of the transferred lymphocytes was suggested by the accumulation of myocardial infiltrating T lymphocytes of the SCID mice receiving lymphocytes from donor cells. Thus, it may be that an in vivo model of autoimmune post-viral myocarditis was demonstrated in SCID mice.

We had already reported that cytotoxic T cell activity was increased in the chronic stage of CB3 myocarditis [19,20]. That is, cytotoxic activities against cultured myocytes assayed by $^{51}$Cr-release method were demonstrated by splenocytes cultured from myocarditis mice. In the current study, it was clearly shown that not only splenocytes obtained from mice with chronic myocarditis but also long-term cultured lymphocytes from myocarditis specimens caused myocardial damage in vitro.

The SCID mouse was used for transfer experiments in several autoimmune diseases [14–17]. Although experimental data from animal models of viral myocarditis support a T cell-mediated mechanism and those from in vitro models of myocardial damage reveal the T cell-mediated cytotoxic reaction [19], direct evidence for autoimmune reaction in vivo in post-viral myocarditis has not been established. The major advantage of the transfer of T and B lymphocytes into SCID mice is that with this model, the pathogenic significance of the transferred cells can be tested directly.

Up to now, three disparate hypotheses have been proposed to explain the basic mechanism of cardiotropic virus-induced ongoing inflammation in murine heart tissues [2–4]. (1) Persistence of virus activities in heart tissues promotes dysregulation or dysfunction of myocytes with release of proinflammatory cytokines [22], (2) virus infection induces myocyte injury which exposes neoanti-
gens that stimulate synthesis of immune effector cells or sensitized T cells [19], and (3) molecular mimicry involving epitopes shared among viral capsid proteins and cardiac contractile proteins on the surface of heart cells stimulates autoimmune reactions [23,24].

Many investigators have supported the importance of cellular immunity in CB3 myocarditis. We had already demonstrated that the exogenously transferred and same-virus (antigen) sensitized T cells are accumulated into damaged myocardium in nude (T cell deficient) mice with CB3 myocarditis and that these cells can cause newly developed ongoing myocarditis [11]. However, direct evidence for autoimmune post-CB3 myocarditis in vivo has not been obtained. We have shown here, for the first time, in vivo evidence of antigen-sensitized lymphocyte-mediated myocardial injuries by adoptive transfer of splenocytes from mice with chronic CB3 myocarditis. The reason why CD4 cells have exerted the cytotoxic activities in vivo may be the immature nature of CD4 T cells, which have both CD4 and CD8 cell types functionally [13].

To date there were two animal models of in vivo autoimmune myocarditis reported in the literature. Kodama et al. reported a giant cell myocarditis in rats caused by the immunization of porcine cardiac myosin, which could be transferred adoptively to an intact rat [25–31]. Another model was reported by Schwimmbeck et al. [6]; they successfully transferred human myocarditis into SCID mice by the intraperitoneal injection of peripheral lymphocytes of patients with myocarditis. Our model of autoimmune myocarditis in post-CB3 myocarditis also needs the SCID mouse background to create in vivo. The reason why active myocarditis could not be transferred in wild mice remains unknown. Because myocardial damage was not detected in normal uninfected recipients of wild mice, the presence of degenerative myocardium or neoantigen-presenting myocardium seems to be necessary for the induction of myocarditis by the current transfer method in normal immune status. In other words, when an immune regulatory system against autoreactive T cells is disturbed by challenge of foreign and mimic antigens, the host of myocarditis with post-viral or unknown etiologies may suffer from recurrence of ongoing autoimmune myocarditis.

The possibility of the so-called persistent infection of CB3 in transferred splenic lymphocytes, which is a well-known phenomenon in CB3 biology [32,33] was ruled out by PCR study.

In conclusion, the SCID mouse model with autoimmune myocarditis appears to be an appropriate in vivo tool to study the significance of immune reactions which might operate in mice with post-CB3 myocarditis. Myocarditis could be transferred into SCID mice by the adoptive transfer of the antigen-sensitized lymphocytes possessing the in vitro cellular cytotoxic activities. An in vivo model of autoimmune post-viral myocarditis in SCID mouse was clearly demonstrated.

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