Sensitive diagnosis of cardiac allograft rejection by detection of cytokine transcription in situ

Jun-ichi Suzuki, Mitsuaki Isobe*, Satoshi Yamazaki, Shiro Horie, Yoshio Okubo, Morie Sekiguchi

The First Department of Internal Medicine, School of Medicine, Shinshu University, Matsumoto, Japan

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Abstract

Objective: In situ transcription of cytokines which are important in the development of cardiac rejection has not been evaluated for diagnosing rejection. The objective was to evaluate the usefulness of in situ reverse transcriptase polymerase chain reaction (RT-PCR) for sensitive detection of acute cardiac rejection.

Methods: We studied interferon (IFN)-γ and interleukin (IL)-2 expression using immunohistochemistry and in situ RT-PCR in murine cardiac transplant models. Hearts were heterotopically transplanted (BALB/c to C3H/He) and some mice were not treated (n=23); others were treated with anti-intercellular adhesion molecule (ICAM)-1 and anti-lymphocyte function associated antigen (LFA)-1 monoclonal antibodies (mAbs) (n=23). Allografts were removed at days 1 to 7. For control, isografts were harvested at day 7 (n=2).

Results: Mice without treatment rejected allografts within 7 days, while all mAb-treated recipients accepted allografts for the same period. At day 1, allografts of both groups showed scattered myocardial cell infiltration which increased in non-treated allografts, but remained stable in mAb-treated grafts thereafter. In situ RT-PCR showed that IL-2 and IFN-γ mRNA positive cells were present in non-treated allografts, while few mRNA positive cells were expressed in infiltrating cells in the mAb-treated allografts (IL-2, day 3: 88.8±28.3 vs. 7.2±6.4, p<0.05, positive cells within 10 fields per section). However, immunohistochemistry could not reveal the difference at day 3. Conclusion: In situ RT-PCR is a sensitive method for diagnosing acute rejection, and it reveals the characteristics of myocardial infiltrate cells to determine their role in the process of rejection.

Keywords: Mouse; Heart transplant; Rejection; Cytokine; Transcription

1. Introduction

Cardiac transplantation as a clinical treatment for end-stage cardiac disease has been performed for more than a quarter of a century [1,2]. Recent studies suggest that cytokines play an important role in regulating immunological responses to transplantation [3,4]. Interferon gamma (IFN-γ) and interleukin (IL)-2 are known to be produced by type 1 helper T (Th1) cells, an essential factor in the progression of rejection [5–9].

Currently, cardiac rejection is mainly diagnosed by pathological observation of myocardial cell infiltration, however, all types of cell infiltration may not indicate acute rejection. For instance, the ‘guilty’ effect refers to the infiltration of the endocardium by lymphocytes, and does not indicate the progression of acute rejection [2]. Therefore, methods for determining the characteristics of the cell infiltrate responsible for acute rejection are needed.

Reverse transcriptase polymerase chain reaction (RT-PCR) allows enzymatic amplification of small amounts of mRNA, but can not identify the cells which express mRNA expression [10]. In situ RT-PCR has recently been developed, and is ideal for immunopathological analysis because it can sensitively identify mRNA positive cells in small tissue specimens [11].

We have reported a cardiac allograft tolerance between fully incompatible mice strains after short-term administration of monoclonal antibodies (mAbs) to intercellular adhesion molecule (ICAM)-1 and lymphocyte function associated antigen (LFA)-1. This tolerance is specific to donor alloantigens, inasmuch as the mice accepted donor-
syngeneic skin, whereas they normally reject third-party skin [12]. While the characteristics of infiltrate cells have not been determined in detail, myocardial cell infiltrates were observed in the accepted allografts during the early stages of the induction of tolerance; cell infiltration remained stable and no myocardial damage was observed in mAb-treated allografts thereafter [7,8], thus demonstrating that infiltrate cells are not the only factor in the progression of acute rejection.

In this report, in situ RT-PCR clarified the different characteristics of these cell infiltrates; Th1 cytokine mRNA expression was enhanced in non-treated allografts but was not expressed in mAb-treated allografts, although myocardial cell infiltration occurred in both groups.

2. Methods

2.1. Animals

Male BALB/c (H-2d) mice (age 4–6 weeks, 20–25 g) and male C3H/He (H-2k) mice (age 4–6 weeks, 20–25 g) were obtained from Japan Charles River Laboratories (Tokyo, Japan). They were housed under conventional conditions and fed a standard diet and water.

2.2. Monoclonal antibodies

The mAbs for tolerance induction used in this study, KBA (IgG2a) and YN1/1.7 (IgG2b) were rat mAb to mouse CD11a (alpha chain of LFA-1) and ICAM-1 respectively (by Prof. Ko Okumura, Juntendo University). Hybridoma cell lines were cultured in RPMI 1640 supplement with 10% fetal bovine serum and 0.1% gentamycin. The mAbs from ascites produced in nude mice were purified with the use of a protein G affinity column [12].

2.3. Heterotopic cardiac transplantation

Donor hearts were heterotopically transplanted into recipient mice by a microsurgical technique [12,13]. Operation time averaged approximately 60 min and the overall success rate was greater than 90%. Because of the incompatibility throughout the H-2 complex, C3H/He mice without any treatment invariably rejected transplanted BALB/c heart allografts within 10 days. Isografts (C3H/He to C3H/He) were removed for examination at day 7 (n=2). Allografts from non-treated mice were removed at days 1 (n=5), 3 (n=5), 5 (n=7) and 7 (n=6). The allografts from mice with anti-ICAM-1 plus anti-LFA-1 mAb treatment (50 μg/day/mouse) were removed at days 1 (n=5), 3 (n=5), 5 (n=7) and 7 (n=6) [14,15].

2.4. Immunohistochemistry

Frozen serial sections were cut (6–8 μm) and incubated with the biotinylated primary antibodies against IFN-γ and IL-2 (PharMingen, San Diego, CA) for 12 h at 4°C in moist chambers. The antibody–biotin conjugates were detected with an avidin–biotin–horseradish peroxidase complex (Nichirei, Tokyo, Japan) according to the manufacturer’s instructions. Enzyme activity was detected with diaminobenzidine (0.5 mg/ml) with 0.05% NiCl in 50 mM Tris buffer, pH 7.5, and sections were counter-stained with Mayer’s hematoxyline [16,17].

2.5. In situ RT-PCR

Frozen sections were placed on silane-coated glass slides and were fixed in 4% buffered paraformaldehyde for 20 min. Protease and DNase digestion were performed as described [18]. Oligoprimers of IFN-γ and IL-2 used in this study have been described [3]. Reverse transcription (RT) solution was prepared as described [19]. The RT solution was applied to each slide and incubated at 42°C for 1 h. PCR solution contained 3 pM sense and antisense cytokine primers each and 5 μl of 10x DIG DNA labeling mix (Boehringer Mannheim, Indianapolis, IN) was applied to the tissue section [19]. The amplification reaction sequence was 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, 30 cycles with the final extension at 72°C for 12 min. Slides were washed in room temperature 5x sodium citrate buffer (SSC), 2x SSC, 1x SSC, and 0.1 x SSC for 5 min. each. The digoxigenin-labeled cDNA segments were detected by enzyme-linked immunoassay using anti-digoxigenin-AP, Fab fragments (Boehringer Mannheim). An enzyme-catalyzed color reaction with 5-bromo-4-chloro-3-indolyl phosphate (x-phosphate) and nitroblue tetrazolium salt (Boehringer Mannheim) produced an insoluble blue precipitate that identified the location of the amplified cytokine cDNA molecules [18,19]. Positive control (no DNase digestion) and negative control (no RT reaction) were also examined [11].

2.6. Quantitation of immunostaining and in situ RT-PCR

A blind analysis of histology specimens was performed by two independent reviewers, and a consensus obtained. Grading of rejection was based on standardized criteria depending on the amount of lymphocytic infiltration [20]. Rejection was graded from 0 to 3 in increments of 0.5 as follows: 0 = no infiltrates; 1 = mild infiltration of lymphocytes; 2 = moderate infiltration; 3 = severe, extensive lymphocytic infiltrates, often with myocytic damage. The cytokine mRNA positive and immunohistochemically positive cells within 10 high-power fields (x400) per section per mouse were counted. One high-power field is 86 μm² and we counted the positive cells with the aid of an ocular grid micrometer.

2.7. Statistical analysis

All data are expressed as mean±SD. Comparisons
between groups were determined using the Student’s t-test for differences in means. A two-sided value of \( p < 0.05 \) was considered statistically significant.

3. Results

Allografts stained with hematoxylin and eosin (HE) from non-treated mice initially showed focal mononuclear cell infiltrates without myocyte damage at day 1; the number of infiltrate cells gradually increased and myocyte damage progressed over a period of time following transplantation. In contrast, myocardial interstitium from cardiac allograft of anti-ICAM-1 and anti-LFA-1 mAb-treated mice initially showed mononuclear cell infiltration at day 1; the cell infiltration remained while myocyte damage did not progress thereafter (Figs. 1 and 2 Table 1).

IFN-\( \gamma \) mRNA expression was initially observed in allografts from non-treated mice at day 1, although immunohistochemistry showed few IFN-\( \gamma \) producing cells in the infiltrates at that point. At day 5 and thereafter, immunohistochemistry showed IFN-\( \gamma \) producing cells in the non-treated allografts, while IFN-\( \gamma \) mRNA expression was severely enhanced (Fig. 3). Conversely, although mononuclear cell infiltration was observed from day 1, immunohistochemistry and in situ RT-PCR revealed that IFN-\( \gamma \) expression was not enhanced in infiltrates in the mAb-treated allografts during the course of the experiments (Fig. 4). In situ RT-PCR revealed that IFN-\( \gamma \) mRNA positive cells were initially observed prior to IL-2 mRNA positive cells, while the number of IL-2 mRNA positive cells was relatively much larger than that of IFN-\( \gamma \) mRNA positive cells in the mononuclear cell infiltrate in the allografts from non-treated mice at day 7 (Fig. 5).

Isografts kept beating for the duration of observation and the isografts were free from signs of rejection. In the isograft controls, immunohistochemistry and in situ RT-PCR revealed at day 7 that few mononuclear cells had infiltrated the myocardial interstitium, and no cytokines were expressed (data not shown).

In in situ RT-PCR positive control studies, blue sign was observed in all cells (sample sections without DNase digestion), while in negative control samples (those without RT step) no blue sign positive cells were seen (data not shown).

4. Discussion

4.1. Characterization of infiltrate cells reveals development of rejection

Cardiac rejection is mainly diagnosed by pathological observation of myocardial cell infiltration, however all cell

![Fig. 1. Types of treatment and numbers of IL-2 or IFN-\( \gamma \) positive cells which were counted within 10 high-power fields per section in sections of immunohistochemistry or in situ RT-PCR are demonstrated. N=No treatment, T=mAb-treatment, IHC=immunohistochemistry, ISPCR=in situ RT-PCR. *\( p < 0.05 \) non-treated group vs. mAb-treated group.](image-url)
Fig. 2. Microscopic findings of cardiac allografts with HE stain. Panels in the upper row (A, B) show cardiac allografts from non-treated mice and the lower row (C, D) are anti-ICAM-1 and anti-LFA-1 mAb-treated allografts. Left panels (A, C) show myocardial cell infiltrates in allografts at day 1; right panels (B, D) are at day 7. No significant difference in scattered cell infiltration was observed between non-treated allografts (A) and mAb-treated allografts (C) at day 1. However, significant cell infiltration was observed in the non-treated allografts (B), while cell infiltration had not progressed in the mAb-treated allografts (D) at day 7 (original magnification: x100).

Infiltration may not indicate acute rejection; the presence of other cell infiltrates, such the ‘guilty’ effect may sometimes lead to a false diagnosis of acute cardiac rejection. Early and precise diagnosis of acute rejection is critical because intensive immunosuppressive treatment should begin before myocardial damage occurs. Therefore, new methods for determining the characteristics of myocardial cell infiltrates are needed for diagnosis of cardiac rejection.

In this study, myocardial cell infiltration was observed soon after transplantation in non-treated and mAb-treated mice, but the prognosis differed significantly between the groups; non-treated grafts were rejected and mAb-treated were accepted. Pathological observation of tissues stained with HE did not reveal the different characteristics of the mononuclear cell infiltration between the groups at any time. Immunohistochemistry, although able to differentiate among cell infiltrates by detecting Th1 expression, does not have the required sensitivity to facilitate early discrimination among infiltrates during the critical period immedi-

**Table 1**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Days after transplantation</th>
<th>1</th>
<th>3</th>
<th>5</th>
<th>7</th>
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<tr>
<td>Score of Rejection</td>
<td>N</td>
<td>0.2±0.3</td>
<td>0.9±0.3</td>
<td>1.9±0.5*</td>
<td>2.3±0.4*</td>
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<tr>
<td></td>
<td>T</td>
<td>0.3±0.3</td>
<td>0.6±0.2</td>
<td>0.9±0.4</td>
<td>1.0±0.4</td>
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<tr>
<td>IFN-γ</td>
<td>N</td>
<td>0.6±0.6</td>
<td>11.4±4.7*</td>
<td>10.1±4.3*</td>
<td>15.8±8.4*</td>
</tr>
<tr>
<td>(IHC)</td>
<td>T</td>
<td>0.6±0.5</td>
<td>3.0±1.2</td>
<td>4.4±2.3</td>
<td>5.6±4.3</td>
</tr>
<tr>
<td>IFN-γ (IS-RT-PCR)</td>
<td>N</td>
<td>36.0±14.8*</td>
<td>65.8±30.9*</td>
<td>125.0±60.6*</td>
<td>87.0±27.5*</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>10.2±6.9</td>
<td>7.3±4.7</td>
<td>9.0±5.5</td>
<td>14.4±11.7</td>
</tr>
<tr>
<td>IL-2</td>
<td>N</td>
<td>0.6±0.9</td>
<td>5.4±2.9</td>
<td>23.6±9.0*</td>
<td>36.7±10.4*</td>
</tr>
<tr>
<td>(IHC)</td>
<td>T</td>
<td>1.0±1.0</td>
<td>3.5±3.5</td>
<td>10.0±3.8</td>
<td>10.4±6.8</td>
</tr>
<tr>
<td>IL-2 (IS-RT-PCR)</td>
<td>N</td>
<td>15.2±13.7</td>
<td>88.8±28.3*</td>
<td>143.4±53.1*</td>
<td>196.3±90.3*</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>8.6±5.9</td>
<td>7.2±6.4</td>
<td>13.8±6.6</td>
<td>19.8±8.1</td>
</tr>
</tbody>
</table>

N=No treatment, T=mAb-treatment, IHC=immunohistochemistry, IS-RT-PCR=in situ reverse transcriptase polymerase chain reaction.

*p<0.05 non-treated group vs. mAb-treated group.
Fig. 3. Microscopic findings of cardiac allografts indicating IFN-γ mRNA positive cells using in situ RT-PCR. Panels in the upper row (A, B) show cardiac allografts from non-treated mice and the lower row (C, D) anti-ICAM-1 and anti-LFA-1 mAb-treated allografts. Left panels (A, C) show myocardial cell infiltrates in allografts at day 1; right panels (B, D) at day 7. Many IFN-γ mRNA positive cells were observed in the scattered cell infiltration in the non-treated allografts at day 1 (A), while few were observed in the mAb-treated allografts on the same day (C). At day 7 (B, D), a disproportionate increase in infiltrates was seen in non-treated allografts compared to those with mAb treatment (original magnification: x400).

ately following transplantation. The superior sensitivity of in situ RT-PCR allowed detection of cytokine mRNA in the early stages of rejection, prior to the detection of cytokine production by immunohistochemistry, when the allografts from both groups showed no difference in the amount of cell infiltration [21].

4.2. Th1 cytokine mRNA in situ expression clarifies development of rejection

Th1 cytokines have been shown to play an important role in immunological regulation of rejection. IL-2 plays a central role in amplification of immune responses to rejection [22,23]. Cyclosporine and tacrolimus, potent immunosuppressants, have been shown to suppress T cell proliferation by inhibiting IL-2 transcription [24,25]. In our immunohistochemical observation, IL-2 producing cells were present in rejected allografts, suggesting that their production demonstrates one of the immunological characteristics of rejection. However, the immunohistochemical information was insufficient for a detailed analysis of the immunological mechanism of the rejection; the population of cytokine producing cells was inadequate and there was a delay between the sequence of alloantigen stimulation and cytokine production. Only 3 days after transplantation, in situ RT-PCR clearly demonstrated that the amount of IL-2 producing cells differed between rejected and tolerated allografts, while immunohistochemistry could not reveal the difference at the point. It was not until day 5 after transplantation that immunohistochemistry was able to demonstrate this difference. In addition, in situ RT-PCR also demonstrated different amounts of IFN-γ expressing cells between the two groups at day 1; immunohistochemistry could not reveal the difference. After day 3, immunohistochemistry also became to distinguish the elevated level of IFN-γ producing cells as well as in situ RT-PCR. These results indicate that in situ RT-PCR is more sensitive than immunohistochemistry to the subtle changes which are initial sign of rejection.

4.3. In situ RT-PCR reveals the immune mechanisms of acute rejection

In situ RT-PCR is valuable not only in diagnosing rejection but also in analyzing the immune mechanisms of rejection. IFN-γ mRNA expression was observed within 1 day after transplantation, while IL-2 was mainly observed 3 days after transplantation. The number of IL-2 mRNA expressing cells was much greater than that of IFN-γ expressing cells after day 3. These findings may be due to differing cytokine characteristics. IFN-γ promotes the proliferation of Th1 cells, since IFN-γ itself acts as a powerful stimulator of naive T cells toward the Th1 subset which expresses IL-2 [26]. IL-2 is a broad stimulator not
Fig. 4. Microscopic findings of cardiac allografts indicating immunohistochemically IFN-γ positive cells. Panels in the upper row (A, B) show cardiac allografts from non-treated mice and the lower row (C, D) anti-ICAM-1 and anti-LFA-1 mAb-treated allografts. Left panels (A, C) show myocardial cell infiltrates in allografts at day 1; right panels (B, D) at day 7. No IFN-γ positive cells were observed in the scattered cell infiltration in the non-treated allografts (A) and the mAb-treated allografts at day 1 (C). However, immunohistochemically IFN-γ positive cells were observed in the marked cell infiltrates (B), while scattered cell infiltrates showed no IFN-γ positive cells (D) at day 7. An arrow in the panel B indicates an immunohistochemically IFN-γ positive cell (original magnification: x400).

Fig. 5. Microscopic findings of cardiac allografts from non-treated mice. Panels in the upper row (A, B) show IFN-γ mRNA positive cells and the lower row (C, D) indicates IL-2 mRNA positive cells using in situ RT-PCR. Left panels (A, C) show the myocardium of an allograft at day 1; the right (B, D) at day 3. IFN-γ mRNA positive cells were initially observed in the allografts from non-treated mice at day 1 (A), prior to the appearance of IL-2 mRNA positive cells at day 3 (D). The number of IFN-γ mRNA positive cells gradually increased (A to B); while that of IL-2 mRNA positive cells significantly increased (C to D) (original magnification: x400).
only of T cells but also of B cells, NK cells, monocytes and macrophages [5,6]. Therefore, IL-2 transcripts may be enhanced by IFN-γ stimulation and the enhanced expression of IL-2 may contribute to extensive stimulation to activation of not only T cells, but also other kinds of cells.

In conclusion, in situ RT-PCR is a sensitive method for diagnosing acute rejection. In situ cytokine mRNA expression reveals the specific characteristics of myocardial infiltrate cells, thus allowing their precise roles in the rejection process to be determined.

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