The Impact of FK506 on Graft Coronary Disease of Rat Cardiac Allograft — A Comparison with Cyclosporine A —
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Abstract

We studied the impact of FK506, a potent immunosuppressant, on graft coronary disease and graft infiltrating lymphocyte subset following rat heart transplantation. Fisher heart grafts transplanted into Lewis recipients were divided into 3 groups: Control (n=7), treated with FK506 at a dose of 0.32mg/kg/day i.m. (n=7), treated with Cyclosporine A at a dose of 10mg/kg/day i.m. (n=7). Grafts were removed on the 71st day in the treated groups and on rejection in the control. We blindly scored graft rejection and graft coronary disease on a scale of 0-4. Graft infiltrating lymphocytes were investigated by flowcytometric analysis using the following monoclonal antibodies: W3/25; anti-helper T lymphocyte, OX8; anti-suppressor & cytotoxic T lymphocyte and OX39; anti-interleukin 2 receptor. There was no difference of graft rejection between two treated groups (FK506 1.66±0.49 vs Cyclosporine 1.45±0.37), but FK506 group expressed severe graft coronary disease (FK506 2.14±0.82 vs Cyclosporine 0.78±0.17 p<0.01) in this model. In flowcytometric analysis, we found an increased proportion of OX8-positive lymphocytes (FK506 25.7±6.4% vs Cyclosporine 4.9±2.4% p<0.01). These results suggest that suppressor of cytotoxic T lymphocytes may be involved in graft coronary disease.

Abbreviated Title: GCD under FK506 in Rat
FK506 is a macrolide antibiotic, which has strong immunosuppressive potential. The immunosuppressive mechanism of FK506 is known to suppress the synthesis of interleukin 2 and the activation of helper T lymphocyte, similar to Cyclosporine A. 1-3 Also FK506 may induce donor-specific suppressor T lymphocyte in rat heart transplantation. Ochiai et al reported FK506 can induce graft tolerance. 4 Thus FK506 is expected to be applied in the near future.

In long term follow up of orthotopic heart transplant patients, new problem of rapidly progressive graft coronary disease (GCD) became apparent. The pathogenesis of GCD remains unknown. But GCD is supposed to be a form of immunological reaction in the chronic phase of heart transplantation.

We investigated the impact of FK506 on graft coronary disease compared with Cyclosporine A and the process of GCD using flowcytometric analysis of lymphocyte-surface markers.

MATERIALS AND METHODS

Transplantation

For this study, two different strains of male inbred rats were selected, Lewis (LEW)(RT11) as recipients and Fisher (F344)(RT11vl) as donors. They were purchased from Charles-River Japan Co.(Atugi,Japan) All animals received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Science and published by National Institutes of Health(NIH Publication No. 86-23 revised 1985).

Heterotopic intraabdominal heart transplantation was performed as Ono and Lindsay described. 5 Fisher rat was first anesthetized by inhalation of ether. After median laparotomy, the donor rat was heparinized at a dose of 1mg/kg from inferior vena cava. Anterior thoracotomy was followed by a bolus injection of St.Thomas cardioplegic solution from inferior vena cava. Afterward cardioplegia was injected from descending aorta and cardiac arrest was accomplished. Procurement of the graft heart was completed by division of ascending aorta and pulmonary artery and ligation-division of the vena cavae & left atrium. The graft heart was immersed in cold saline. Recipient Lewis rat was anesthetized by ether inhalation. The median laparotomy was performed and abdominal aorta and inferior vena cava were dissected freely beneath their renal branches. Abdominal aorta & inferior vena cava were cross-clamped independently. Aortoaortic end-to-side anastomosis and pulmonocaval end-to-side anastomosis were performed by 8-0 Prolene continuous sutures. Mean ischemic period was approximately 60 minutes.

Transplanted rats were divided into 3 groups as follows: control without immunosuppression (n=7), treated with Cyclosporine A at a dose of 10mg/kg/day i.m.(n=7). and treated with FK506 at a dose of 0.32mg/kg/day i.m.(n=7). The immunosuppressants were injected intramuscularly in alternate legs daily for 70 days. Pulsation of the graft was confirmed by daily palpation and the cessation of pulsation was diagnosed as graft rejection.

All grafts of control group were rejected and mean graft
survival was 10.3 days. Grafts were removed on the day of the rejection. Grafts of the immunosuppressed groups achieved long-term survival more than 70 days and sacrificed by terminal ether anesthesia on the 71st day. The removed grafts were evaluated for graft rejection, graft coronary disease and surface markers of lymphocytes by the following methods.

Histological Examination

The basal half of the graft heart was fixed by formaldehyde for microscopic examination. Graft rejection was graded according to International Society for Heart Transplantation(ISHT) Standardized Grading System by the Hematoxylin-Eosin stained specimen. Graft coronary disease was graded by the Elastica van Gieson stained specimen as follows; grade 0: no stenosis, 1: 1-25% stenosis, 2: 25-50%, 3: 50-75%, 4: 75-100%. Both evaluations were carried out blindly.

Flowcytometric Analysis

Lymphocytic surface marker analysis by flowcytometry, using EPICS model 753: Coulter Electronic Co.(Hialeah,Florida U.S.A), was performed on both peripheral blood lymphocytes(PBL) and graft infiltrating lymphocytes(GIL).

PBL Preparation: PBL were prepared from heparinized blood using Lympholyte-R:Cederlane Lab.(Hornby,ON, Canada). Blood was overlaid on Lympholyte-R and centrifuged at 2200 r.p.m. for 30 minutes in room temperature. The layer of lymphocytes was carefully aspirated. Lymphocytes were suspended in RPMI 1640 culture medium containing 1% of fetal calf serum and 1% of 0.1% NaN₂.

GIL Preparation: The apical half of the graft heart was minced by a tissue homogenizer and cells were released according to Totterman et al described. The tissue homogenate was incubated with 3ml of digestion medium (20mM Hepes Buffer from Sigma, 130mM NaCl, 4.7mM KCl, 0.65mM MgSO₄, and 1.2mM CaCl₂, pH 7.45) containing collagenase 2mg/ml, DNAse 0.05mg/ml and 1.5% bovine serum albumin. After agitation and incubation at 37°C for 1 hour, cells were filtrated through nylon mesh (100μm) to remove aggregates and overlaid on Lympholyte-R. Thereafter centrifugation at 2200 r.p.m. for 30 minutes made a clear lymphocytic layer and these cells were carefully aspirated and suspended in the same medium as PBL.

Antibody Incubation: The lymphocyte suspensions of the GIL and PBL were incubated with the optimal concentration of monoclonal antibodies at 4°C for 30 minutes in a dark incubation chamber. Lymphocyte-surface markers examined were W3/25: anti-helper T lymphocyte antibody labeled with fluorescein(FITC), OX8: anti-suppressor & cytotoxic T lymphocyte antibody labeled with phycoerythrin(PE), OX39: anti-interleukin 2 receptor antibody labeled with PE. Two combinations of the antibodies, W3/25 with OX8 and W3/25 with OX39, were stained doubly and used for two color flowcytometry analysis. The cells were gated optimally by forward scatter (cell size) and lateral scatter (granularity) for lymphocyte. Appropriate filters, 560nm short pass filter for FITC and 590nm long pass filter for PE, were used in the analysis.

All of the statistical analysis was performed using the ANOVA test.
Results

Graft rejection of the control group was severe, compatible with ISHT grade 4. FK506 group and Cyclosporine A group showed mild to moderate rejection respectively and their grading did not differ significantly (Figure 1).

In respect of graft coronary disease, the control group was almost free of coronary stenotic lesions. Both FK506 and Cyclosporine A caused GCD and their subquantitative evaluation revealed significant difference between the two groups. FK506 group showed more severe GCD than seen in Cyclosporine A treated grafts (Figure 2).

The flowcytometric analysis of the lymphocytic surface markers showed an interesting difference between the three groups in terms of graft infiltrating lymphocytes.

First the proportion of W3/25 positive cells in the GIL, the helper T lymphocyte subpopulation, in the FK506 group was significantly greater than in the control group and significantly lower than Cyclosporine A group. (Figure 4)

The OX8 positive cell proportion in the GIL, the cytotoxic and suppressor T lymphocyte subpopulations, in the FK506 group was significantly lower than the control group but greater than the Cyclosporine A group (Figure 5).

OX39, which binds to the interleukin 2 receptor of activated lymphocytes showed no statistically significant difference among the three groups in the GIL evaluation (Figure 6).

These data suggest that the non-treated rejector had the most intense CD8 lymphocytic infiltrate among the three groups and that the FK506 treated graft had a different GIL population than the Cyclosporine A treated grafts.

In the flowcytometric analysis of the peripheral blood lymphocytes, none of these markers were significantly different among the three groups.

DISCUSSION

FK506 has been reported to prolong allograft survival in different species of animals. Rat heart allograft survival was reportedly achieved by a short course of treatment in the acute phase of rejection without any serious adverse effect. 8-10 But there were few reports on chronic administration of FK506 in rat heart transplantation. Our report focuses on how maintenance use of FK506 affects rat heart allograft, especially in terms of graft coronary disease. In clinical heart transplantation, graft coronary disease has turned out to be an important factor in determining transplant outcome. Narrod et al reported an incidence of 66% of graft coronary disease 6 years after heart transplantation, but its pathogenesis remains unknown. 11-14 If FK506 will be applied into clinical heart transplantation, its impact on graft coronary disease must be carefully evaluated.

Our experiment revealed FK506 at a dose of 0.32mg/kg/day i.m., which may be a relatively high dose, was associated with more progressive graft coronary disease than Cyclosporine A. Vascular inflammatory changes were reported in canine renal transplantation treated with FK506. 15 But there have been no reports of vascular changes in the rat with administration of
FK506\textsuperscript{16-17} and we have not observed any vascular changes in the native heart, lung kidney and aorta of both recipients and rats without allografting receiving 0.32 mg/kg/day i.m. daily for 70 days. And Meiser et al. also reported FK506 caused severe graft coronary disease in rat allotransplantation model.\textsuperscript{18} So it is appropriate to consider the coronary stenotic lesions observed in our experiment to be the expression of graft coronary disease and not a partial expression of the generalized vascular adverse effect of FK506.

Flowcytometric analysis of surface markers on the graft infiltrating lymphocytes reflects the rejection process more accurately than the same analysis on peripheral blood lymphocytes and quantitatively. Our method of the preparation for graft infiltrating lymphocytes was highly efficient and specific, and thus the results were reproducible and excellent for immunological analysis of the intragraft event. So we expect our methods can be useful for diagnosis of rejection in the clinical practice. In this study we examined the T lymphocyte subsets, which infiltrated grafts under chronic FK506 and cyclosporine immuno-suppression. We found the suppressor and cytotoxic T(CD8) lymphocyte proportion of the FK506 group to be increased significantly compared with the Cyclosporine A group. Woo et al reported reduction of W3/25:OX8 ratio in splenic lymphocytes in rat stimulated by sheep erythrocytes treated by FK506 and Cyclosporine A. These observations and our results show that FK506 has an OX8 positive cell induction effect.\textsuperscript{19-21}

Rejection grade of the two groups were not different and interestingly the graft coronary disease of the FK506 group was more advanced than in the Cyclosporine A group. These results lead us to the idea that the cytotoxic or suppressor T lymphocyte was involved in the graft coronary disease. Macrophages and smooth muscle cells were known to exist in lesions of graft coronary disease.\textsuperscript{22} But immunoreaction of these cells was not alloantigen-specific and these cells exist in the atherosclerotic lesions, therefore expression of macrophages and endothelial proliferation may only be the terminal result of endothelial injury. Therefore, we speculate that cytotoxic T lymphocytes play a role in initiation and proliferation by recognizing and destroying graft vascular endothelium, which thus results in graft coronary disease. And we have to evaluate further, the localization of the antigens, multiple doses studies, the adoptive graft coronary disease model and clinical evaluation of CD8 antigen in biopsy specimens and their correlation with graft coronary disease, to prove this hypothesis.

FK506 is one of the most potent immunosuppressants that can be applied in clinical transplantation. However we have observed its possible adverse impact on graft coronary disease with this experimental model with rat heart transplant. Armitage et al. reported that there has been no realization of vasculitis in clinical experience with FK506 in heart transplantation with median follow-up period of 110 days.\textsuperscript{23} And Flavin et al. reported that cynomolgus monkeys treated with FK506 following heterotopic heart transplantation expressed only a limited incidence of graft coronary disease and also their lesions may be different from the lesions among the rat allografts.\textsuperscript{24} So the graft coronary disease
observed in this study may be unique to the rat heart transplantation model. But we have to carefully examine the impact of FK506 on graft coronary disease in clinical practice and experimental model.

REFERENCES


Figure 1  Graft Rejection
Rejection grade of CsA and FK506 did not differ significantly.
CsA: Cyclosporine A  NS: No Significance

Figure 2  Graft Coronary Disease
Graft coronary disease of FK506 was more advanced than Cyclosporine A.
CsA: Cyclosporine A
Grading Scale of Graft Coronary Disease:
Grade 0: no stenosis, 1: 1-25% stenosis, 2: 25-50%,
3: 50-75%, 4: 75-100%

Figure 3  Graft Coronary Disease Lesion (X 400)
Left picture shows the epicardial lesion of FK506 and right one shows the epicardial lesion of Cyclosporine A. FK506 group expressed more stenotic lesions.

Figure 4  Flowcytometric Analysis
Proportion of W3/25 positive lymphocytes (Th) among graft infiltrating lymphocytes.

Figure 5  Flowcytometric Analysis
Proportion of OX8 positive lymphocytes (Ts/c) among graft infiltrating lymphocytes.

Figure 6  Flowcytometric Analysis
Proportion of OX39 (IL2R) positive lymphocytes among graft infiltrating lymphocytes.

TABLE 1 Grading of graft rejection and graft coronary disease

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose</th>
<th>GR</th>
<th>GCD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=7)</td>
<td>no treatment</td>
<td>4.00±0.00</td>
<td>0.13±0.35</td>
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<tr>
<td>FK506 (n=7)</td>
<td>0.32mg/kg/day i.m.</td>
<td>1.66±0.49*</td>
<td>2.14±0.82**</td>
</tr>
<tr>
<td>CsA (n=7)</td>
<td>10mg/kg/day i.m.</td>
<td>1.45±0.37*</td>
<td>0.78±0.17</td>
</tr>
</tbody>
</table>

GR: graft rejection  GCD: graft coronary disease
CsA: Cyclosporine A
GCD grading: grade 0: no stenosis, 1: 1-25% stenosis, 2: 25-50%
3: 50-75%, 4: 75-100%
Figures stand for mean ± standard deviation
*:p<0.01 vs control  **:p<0.01 vs both control and CsA
TABLE 2 Flowcytometric analysis of graft infiltrating lymphocytes

<table>
<thead>
<tr>
<th>Group</th>
<th>W3/25 (Th)</th>
<th>OX8 (Ts/c)</th>
<th>OX39 (IL2R)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>49.8±19.1</td>
<td>43.3±20.3</td>
<td>52.1±14.3</td>
</tr>
<tr>
<td>FK506</td>
<td>70.5±5.6*</td>
<td>25.7±6.4*</td>
<td>48.4±14.4</td>
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<tr>
<td>CsA</td>
<td>86.9±2.8**</td>
<td>4.9±2.4**</td>
<td>51.7±21.3</td>
</tr>
</tbody>
</table>

CsA: Cyclosporine A
Figures stand for mean ± standard deviation
* : p<0.01 vs Cyclosporine A and p<0.05 vs control
** : p<0.01 vs control

TABLE 3 Flowcytometric analysis of peripheral blood lymphocytes

<table>
<thead>
<tr>
<th>Group</th>
<th>W3/25 (Th)</th>
<th>OX8 (Ts/c)</th>
<th>OX39 (IL2R)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>62.2±8.0</td>
<td>25.7±3.4</td>
<td>26.2±11.0</td>
</tr>
<tr>
<td>FK506</td>
<td>68.1±10.5</td>
<td>22.8±1.2</td>
<td>34.6±14.0</td>
</tr>
<tr>
<td>CsA</td>
<td>73.0±9.4</td>
<td>23.2±8.5</td>
<td>28.0±14.4</td>
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</tbody>
</table>

CsA: Cyclosporine A
Figures stand for mean ± standard deviation