Prolonged Survival in Rat Liver Transplantation with Mouse Monoclonal Antibody Against an Inducible Costimulator (ICOS)

Lei Guo, Xiao-Kang Li, Naoko Funeshima, Masayuki Fujino, Yuiko Nagata, Hiromitsu Kimura, Hiroshi Amemiya, Shin Enosawa, Takashi Tsuji, Yasushi Harihara, Masatoshi Makuchii, and Seiichi Suzuki

Department of Experimental Surgery and Bioengineering, National Children’s Medical Research Center, Tokyo, Japan; Department of Artificial Organ and Transplantation Surgery, Graduate School of Medicine and Faculty of Medicine, University of Tokyo, Tokyo, Japan; and Pharmaceutical Frontier Research Laboratories, JT Inc., Yokohama, Kanagawa, Japan

Background. An inducible costimulator (ICOS), a recently identified costimulatory receptor with a close structural homology to CD28 and CTLA4, is expressed on activated T cells. Interaction with its ligand on antigen-presenting cells stimulates T-cell proliferation to produce a different spectrum of cytokine. The inhibition of ICOS-mediated signal transduction by an anti-ICOS antibody is considered to be capable of protecting against graft rejection in organ transplantation.

Methods. An anti-rat ICOS antibody was intravenously administered into recipients of dark Agouti-to-Lewis liver transplantations. The recipient lymphocytes from mesenteric lymph nodes were harvested on day 7 after transplantation for fluorescence-activated cell sorting analysis, and tissue specimens from the grafts were removed for histologic evaluation. Antigen-specific T-cell proliferation responses were assessed in vitro with anti-ICOS antibody.

Results. Monotherapy with the antibody significantly prolonged the graft survival time by inhibiting T-cell activation and its proliferation response. The graft-infiltrating cells, both CD4 and CD8 T cells, were not completely reduced even when rats were administered the antibody, whereas the expression of ICOS almost completely disappeared in these cells.

Conclusions. T-cell activation through the ICOS costimulatory pathway plays an important role in graft rejection, and manipulating its pathway is an effective method for modulating transplantation immunity.

It has been well established that optimal activation of T lymphocytes and development of adaptive immunity require two signals. Signal 1 is transduced by T-cell receptors with specific antigen recognition; an additional signal, termed signal 2, is transduced by costimulatory molecules (1, 2). On the basis of this two-signal concept, great interest has been focused on manipulating the second signal to modulate T-cell immunity. In previous studies, increasing the strength of the second signal promoted tumor immunity (3); blocking this signal reduced graft rejection and recovery of autoimmune diseases (4). Several successful experiments demonstrated that the costimulator signal plays a critical role in modulating T-cell immunity (5).

It is known that the CD28 family plays a key role in regulating T-cell activation; costimulation of a T cell through CD28 is critical for generating antigen-specific immune responses, including an increase of CD4 T-cell proliferation to enhance the production of cytokines, which induce the maturation of the CD8 effector T cell (3, 6, 7) and promote T-cell survival (8). In contrast, costimulation via CTLA4 down-regulates the immune responses by inhibiting T-cell proliferation, cytokine production, and cell cycle progression (9, 10).

Recent studies discovered that other members of the B7/CD28 family also participate in the regulation of cellular and humoral immune responses. One of these is an inducible costimulator (ICOS) that is also called an activation-inducible lymphocyte immunomediatory molecule (II, 12). Unlike CD28, an ICOS is not constitutively expressed on T cells, but is induced after T-cell activation (13). Despite the structural homology of an ICOS with CD28 and CTLA4 (19% and 13% similarity of amino-acid sequence of an ICOS to CD28 and CTLA4 in rat) (11), the ICOS differs in mechanisms of action from the others; it does not interact with ligands for CD28 and CTLA4 (B7.1 and B7.2). A novel member of the B7 family called B7 h (14), B7RP-1 (15), GL50 (16), or LICOS (17) has been identified as a ligand for ICOSs. An ICOS may enhance basic T-cell responses to a foreign antigen, including cell proliferation, secretion of lymphokines, up-regulation of molecules that mediate cell-cell interaction, effective help for antibody secretion by B cells (13), and maintenance or modulation of memory T-cell function (18). Signals via the ICOS with those from the T-cell receptor increased T-cell proliferation in mice and humans, and preferentially promoted interleukin (IL) 10 production (13, 15). Furthermore, ICOS expression can be enhanced by CD28 costimulation and regulates differentiation of T cells in mice (14, 19). Coyle et al. (5) revealed that an ICOS is an important costimulatory receptor for recently activated cells and for Th2 but not Th1 effector cells. Therefore, inhibition of an ICOS would be ef-

1 This study was supported by research grants from the Ministry of Health, Labour and Welfare of Japan (12-KO-2, Millennium Project H12-Saisei-016), and a Grant-in-Aid (No. 10307030) and a Grant for Organized Research Combination System from the Ministry of Education, Culture, Sports, Science and Technology of Japan.
2 Department of Experimental Surgery and Bioengineering.
3 Department of Artificial Organ and Transplantation Surgery.
4 Pharmaceutical Frontier Research Laboratories.
5 Address correspondence to: Seiichi Suzuki, MD, PhD, Department of Experimental Surgery and Bioengineering, National Children’s Medical Research Center, 3-35-31 Taishido, Setagaya-ku, Tokyo 154-8509, Japan. e-mail: ssuzuki@nch.go.jp
effective in suppressing the function of recently activated T-helper cells to inhibit the secretion of both IL-4 and interferon-γ. Although interaction with CD28/B7 is critical for the initiation of an immune response, that of an ICOS with its ligand is required at a late stage of the response and predominates over CD28 for secondary immune responses (5). The ICOS is also important as an adhesion molecule, although there is a marked difference between CD28 and an ICOS in their adhesion-supporting abilities; CD28, a major costimulator, has a low affinity toward the ligand(s), whereas an ICOS causes strong cell adhesion. This suggests that an ICOS may play an important role in building the "immunologic synapse" (12, 20).

In this study, we investigated the effect of anti-ICOS antibody monotherapy on dark Agouti (DA)-to-Lewis liver transplantation. The results presented here demonstrate that the anti-ICOS antibody significantly prolonged the survival of the liver graft by inhibiting T-cell activation and proliferation.

MATERIALS AND METHODS

Animals and antibody. Adult male Lewis (RT1l) and DA (RT1a) rats, weighing 210 to 250 g, were used as recipients and donors, respectively. The animals were maintained under standard conditions and fed rodent food and water, according to the principle of laboratory animal care and the guide for the care and use of laboratory animals in our institution. Anti-rat ICOS mouse monoclonal IgG1 (JTT.1) was generated by the method described in the previous paper (12).

Orthotopic liver transplantation. Orthotopic liver transplantation was performed without revascularization of the hepatic artery as originally described by Kamada and Calne (21, 22) and Kamada et al. (23). In brief, the donor liver was flushed through the portal vein with ice-cold sterile saline. The liver grafting was initiated by anastomosing the suprahepatic vena cava with 7-0 sutures. Thereafter, anastomosis of the portal vein and infrahepatic vena cava was conducted using the cuff technique (24, 25). As we have previously demonstrated that the onset day of graft rejection is day 3 after grafting in the DA-to-Lewis liver transplantation model (26, 27), the recipients that died within 3 days after surgery in the present study were considered to be technical failures and excluded from the study. The number of exceptions was approximately 5% of all recipients.

Experiment design. The recipients were intravenously injected with anti-ICOS antibody at 0.3 and 1 mg/kg. The injection was conducted once on day 0 after transplantation, twice on days 0 and 6, or every 3 days until 6 to 12 days after grafting. We used an isotype antibody (mouse IgG1) as a control antibody. Experimental groups were conducted once on day 0 after transplantation, twice on days 0 and 6, with anti-ICOS antibody at 0.3 and 1 mg/kg. The injection was conducted using the cuff technique (24, 25). As we have previously demonstrated that the onset day of graft rejection is day 3 after grafting in the DA-to-Lewis liver transplantation model (26, 27), the recipients that died within 3 days after surgery in the present study were considered to be technical failures and excluded from the study. The number of exceptions was approximately 5% of all recipients.

Histologic studies. Liver grafts were harvested on day 7 from group 1 and group 6. Formalin-fixed samples were embedded in paraffin for hematoxylin and eosin (HE) staining and cut into 4-μm-thick sections. The samples were snap-frozen in tetrafluoroethane for immunohistochemical staining and stored at −80°C. A thin cryocut section of the frozen piece was stained with mouse monoclonal antibodies for rat T cells, as previously described (28). Briefly, the slides with sectioned samples were air-dried and fixed in acetone. The slides were incubated with the primary mouse monoclonal antibody specific for rat CD2 (Serotec, Oxford, U.K.), CD4 (Cedarlane Laboratories, Ontario, Canada), or CD8 (BD PharMingen, San Diego, CA) diluted in the working solution (phosphate-buffered saline [PBS] solution containing 2% bovine serum and 0.1% sodium azide) at 4°C overnight. The slides were then incubated for 1 hr with a secondary antibody (anti-mouse IgG) consisting of alkaline phosphatase-conjugated goat antibody (Santa Cruz Biochemicals, Santa Cruz, CA) diluted at 1/100 in the working solution. Color development was performed with an alkaline phosphatase substrate kit (Vector Laboratories Inc, Burlingame, CA). The sections were counterstained with hematoxylin.

Preparation of lymphocytes and flow cytometric analysis. Lymphocytes were prepared from mesenteric lymph nodes by the following method. The lymph nodes were gently ground with frosted objective slides in PBS to obtain a single-cell suspension, and thereafter the cells were overlaid on Ficol Isopaque (Lympholyt-Rat; Cederlane, Ontario, Canada). After centrifugation at 3000×g for 20 min, the cells of the interface layer were harvested and suspended at 2×10⁶/ml in RPMI 1640 medium containing 10% fetal calf serum. The cells were incubated at 4°C for 30 min with a saturating concentration of R-Phcoerythrin (PE)-conjugated mouse anti-rat CD4 antibody (W3/25, BD PharMingen) or CD8 antibody (MCR OX-8, BD PharMingen) in combination with mouse anti-rat CD25 (α chain of IL-2 receptor) antibody (OX-39, BD PharMingen) conjugated with fluorescein isothiocyanate (FITC) or anti-ICOS antibody. We used a secondary antibody, FITC-conjugated anti-mouse IgG, for ICOS-staining. The control cells were incubated with FITC-conjugated mouse IgG. Finally, the cells were suspended in 1 ml of PBS and analyzed with flow cytometry (FACScan, BD PharMingen). The dead cells were excluded from analysis by staining with propidium iodide fluorescence.

One-way T-cell proliferation assay. The proliferation of T cells to alloantigens was measured with cell proliferation ELISA kits (Roche Molecular Biochemicals, Mannheim, Germany). Briefly, lymphocytes (4×10⁵) from Lewis rat spleen were incubated with 20-Gy-irradiated DA splenocytes (2×10⁵) from Lewis rat spleen were incubated with 20-Gy-irradiated DA splenocytes (2×10⁵) in the presence of various concentrations of the anti-ICOS monoclonal antibody and control antibody in a 96-well microtiter plate at a final volume of 100 μl/well in a humidified atmosphere at 37°C for 6 days. The cells were labeled with 5-bromo-2-deoxyuridine (BrdU) solution at 10 μl/well and incubated for an additional 2 hr at 37°C. After centrifugation, we removed the supernatant, added 200 μl/well of fixation and DNA denaturation (FixDenat; Roche Molecular Biochemicals, Mannheim, Germany) solution to the cells and reincubated them for 30 min at 15 to 25°C. We then cultured the cells for 90 min with anti-BrdU-peroxidase solution and subsequently washed them three times. After adding substrate solution at 100 μl/well, we measured the BrdU incorporation with a chemiluminescence reader.

Statistics. A statistical evaluation for graft survival was performed using the Kaplan-Meier test.

Table 1. Graft survival of DA liver in Lewis recipient administered intravenous anti-ICOS antibody

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>n</th>
<th>Survival</th>
<th>Median</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>No treatment</td>
<td>6</td>
<td>10, 11×3, 12×2</td>
<td>11</td>
<td>NS</td>
</tr>
<tr>
<td>2</td>
<td>Isotype IgG1 1 mg/kg D 0, 3, 6, 9, 12</td>
<td>5</td>
<td>11×2, 12×2, 13</td>
<td>12</td>
<td>NS</td>
</tr>
<tr>
<td>3</td>
<td>ICOS 1 mg/kg D 0</td>
<td>5</td>
<td>10×2, 11, 12, 13</td>
<td>11</td>
<td>NS</td>
</tr>
<tr>
<td>4</td>
<td>ICOS 1 mg/kg D 0, 6</td>
<td>5</td>
<td>10, 11×2, 12, 14</td>
<td>11</td>
<td>NS</td>
</tr>
<tr>
<td>5</td>
<td>ICOS 1 mg/kg D 0, 3, 6</td>
<td>7</td>
<td>10, 14, 16, 19, 25, 27, 28</td>
<td>19</td>
<td>S</td>
</tr>
<tr>
<td>6</td>
<td>ICOS 1 mg/kg D 0, 3, 6, 9, 12</td>
<td>9</td>
<td>13, 16, 19×2, 23, 25, 29, 31, 32</td>
<td>23</td>
<td>S</td>
</tr>
<tr>
<td>7</td>
<td>ICOS 0.3 mg/kg D 0, 3, 6, 9, 12</td>
<td>6</td>
<td>12×2, 14, 17, 25, 27</td>
<td>15.5</td>
<td>S</td>
</tr>
</tbody>
</table>

* Compared with group 1. S, P<0.001; NS, not significant.
RESULTS

Allograft survival. Administration of anti-ICOS antibody at 1 mg/kg three or five times at intervals of 3 days (groups 5 and 6) significantly improved the graft survival, when compared with the control group (groups 1 and 2). However, one or two administrations of the antibody did not show any remarkable effect on the survival (groups 3 and 4). When we administered the antibody at 0.3 mg/kg (group 7) five times, we also obtained a significant prolongation of the survival time (Table 1).

Expression of CD25 and ICOS in peripheral lymph nodes. The lymphocytes were prepared from recipient mesenteric lymph nodes on day seven after grafting from groups 1 and 6 and the normal Lewis rats. The expression level of CD25 on the CD4 and CD8 T cells was analyzed by using fluorescence-activated cell sorting (FACS) analysis. The proportions of CD4/CD25 and CD8/CD25 double-positive cells were significantly decreased in group 6 when compared with group 1, but had not reached normal levels (Fig. 1). We also examined the expression of the ICOS on the CD4 and CD8 T cells in these three groups. The normal lymphocytes showed low ICOS expression; CD4/ICOS and CD8/ICOS double positives were 3.7% and 8.3%, which was consistent with the observation by McAdam et al. (29). The ICOS expression was up-regulated on both the CD4 and CD8 T cells in group 1. Treatment with anti-ICOS antibody (group 6) markedly reduced the ICOS expression on both CD4 and CD8 T cells. We found that the ICOS expression on the CD8 T cells in group 6 was reduced almost to the normal level. However, its expression on the CD4 T cells was still higher than in normal cells, even when treated with anti-ICOS antibody. These data suggested that both CD4 and CD8 cells are targets of the antibody; the reduction rate was relatively high in CD8 T cells (Fig. 2).

Inhibition of T cell proliferation. Antigen-specific T-cell proliferation assays were performed in vitro with graded doses of anti-ICOS antibody. T-cell proliferation was completely inhibited in the presence of the antibody compared with the untreated and the control IgG-treated groups. A greater inhibition of cell proliferation was achieved even when we reduced the anti-ICOS antibody to 0.01 μg/ml (Fig. 3). Thus, the anti-ICOS antibody could completely inhibit T-cell proliferation to allogeneic cells.

HE and immunohistochemical staining. The graft biopsy samples on day 7 after grafting in groups 1 and 6 were stained with HE and examined with light microscopy. We observed extensive infiltrated mononuclear cells in both groups 1 and 6; a severe hepatocyte destruction was seen in group 1, but not in group 6 (Fig. 4). These cells in group 1 were shown to be CD2+`, CD4+`, and CD8+` cells by observation of immunohistochemical staining (Fig. 5). The infiltration of CD2, D4, and CD8 cells was also seen in group 6. This indicated that the anti-ICOS treatment did not reduce T-cell infiltration into the liver graft. In contrast, a large number of infiltrated cells expressed ICOS molecules in group 1, although its expression had almost completely disappeared in group 6 (Fig. 5).

DISCUSSION

The recent development of new immunosuppressive agents, such as FK506 and mycophenolic mofetil, has significantly improved graft survival in organ transplantation (30). However, the toxicity of these reagents still limited their use in clinical patients. Another approach to reducing graft rejection by manipulating costimulatory signals to inhibit T-cell activation has been proved to be effective in experimental organ grafting. Among the costimulatory signals, the B7-CD28/CTLA4 and CD40-CD40L pathways are known to be important for modulating lymphocyte responses. Blocking either or both of these pathways markedly prolonged the survival time of heart, islet, and liver allografts and induced tolerance in some cases (31, 32). In the present study, an ICOS-mediated pathway, a newly identified costimulatory signal for T-cell activation, was blocked with anti-ICOS antibody to investigate whether this treatment would lead to prolongation of graft survival time, using a rat liver transplantation model. Our results demonstrated that one or two administrations of anti-ICOS antibody at 1 mg/kg had no effect on graft survival. The antibody injections repeated
more than three times were necessary for prolongation of the survival time.

The precise action mechanism(s) of an ICOS in regulating T-cell activation has remained unclear. Some investigators suggested that the primary response for T-cell activation is largely dependent on CD28, whereas the secondary response is CD28-independent. ICOS-dependent (33) and CD28-dependent T-cell activations at least partially up-regulate the ICOS expression to enhance the T-cell activation and proliferation. ICOS-deficient mice were generated in more recent studies; these mice had impaired germinal center formation, resulting in profound deficits in immunoglobulin isotype class switching (34, 35). The ICOS-negative T cells had defects in T-cell activation and proliferation, and thus failed to produce IL-4 (35, 36).

We analyzed the T-cell population in graft-infiltrated cells and the expression of CD25 and ICOS in the lymphocytes from mesenteric lymph nodes to investigate the mechanism of action of the anti-ICOS antibody. FACS analysis demonstrated that the administration of anti-ICOS antibody significantly decreased CD25 expression in the lymph nodes, although it did not reduce its expression to the normal level. This suggests that the anti-ICOS antibody could down-regulate the activation of T cells. The antibody treatment also decreased the ICOS expression on both CD4 and CD8 T cells in the lymph nodes, in which the reduction rate was marked in CD8 T cells compared with that in CD4 cells.

The graft sections stained with anti-CD2 antibody on day 7 after grafting indicated that the administration of anti-ICOS antibody does not reduce T-cell infiltration. The infiltrated cells expressed both CD4 and CD8 phenotypes similar to those in the control allografts, although the ICOS expression had almost completely disappeared in the antibody-treated group. This suggests that both CD4 and CD8 T cells are targets of the anti-ICOS antibody. However, the antibody treatment did not reduce cellular infiltration in the allograft, although the reduction of ICOS expression in the infiltrated cells may alter the lymphocyte function related to graft rejection. Rosengard et al. (37) also observed that tolerance-induced recipients in class I disparate renal allografts had intensive lymphocyte infiltration in the grafts, but these cells could not give rise to donor-specific cell-mediated lymphocyte toxicity. We previously demonstrated that T-cell activation is dependent on the B7/CD28 pathway correlated with infiltration into the allograft, because blocking the B7/CD28 pathway with CTLA4-Ig markedly reduced cell infiltration in a cardiac graft (38, 39). In addition, the present study indicates that the cytotoxic activity of graft-infiltrating cells is strongly related to ICOS expression.

There is some discrepancy between our studies and those of others regarding the in vitro analysis of ICOS-mediated T-cell activation and proliferation; i.e., our in vitro study demonstrated that the presence of anti-ICOS antibody completely inhibited T-cell proliferation, whereas McAdam et al. (29) indicated that a soluble ICOS (ICOS-Ig) does not block proliferation of CD4+ cells stimulated with antigen peptide and APC in mice. Furthermore, Aicher et al. (19) reported that the blockage of human T-cell proliferation was so limited that the inhibition rate was approximately 50% by in vitro treatment of ICOS-Ig. It should be noted that there are fundamental differences between the studies using ICOS-Ig.
Figure 4. HE staining in the grafts 7 days after grafting. Extensive cell infiltration was seen both in the control allograft (A, group 1) and the anti-ICOS antibody-treated allograft (B, group 6). However, we observed hepatocyte destruction only in group 1.

Figure 5. Immunohistochemical staining for CD2, CD4, CD8, and an ICOS in the grafts 7 days after grafting and in a normal liver. Severe infiltration of CD2, CD4, and CD8 T cells appeared in the perivascular areas in both the treated and untreated group (A–C and E–G, respectively) but not in a naive Lewis liver (I–K). The treatment with anti-ICOS antibody resulted in an almost complete inhibition of ICOS expression in infiltrating cells (H) when compared with an untreated allograft (D). No ICOS-positive cells were observed in the naive Lewis liver (L).
and those using anti-ICOS antibody. First, the target of the reagents used differed; ICOS-Ig combines with its ligand on APC, whereas anti-ICOS antibody binds to the ICOS-receptor of T cells. Second, the expression level of ICOS appears to differ in T cells among mice, humans, and rats (20). Hence, the appreciable differences of ICOS expression by species and the different targets of either the ICOS receptor or its ligand may reflect the diverse effect on T-cell activation and proliferation.

In summary, we demonstrated the effectiveness of the anti-ICOS antibody on hepatic graft survival in rats. The antibody monotherapy significantly prolonged graft survival by inhibiting the activation and proliferation of CD4 and CD8 T cells via reduction of the ICOS expression, primarily in graft-infiltrating cells. In our ongoing study, we have confirmed that a combined treatment using anti-ICOS antibody and FK506 results in an induction of transplant tolerance in rat liver grafting (manuscript in preparation). Thus, it is possible that combination therapy with conventional immunosuppressive drugs may further improve allograft survival.

REFERENCES
