FTY720, a Novel Immunosuppressant, Induces Sequestration of Circulating Mature Lymphocytes by Acceleration of Lymphocyte Homing in Rats. II. FTY720 Prolongs Skin Allograft Survival by Decreasing T Cell Infiltration into Grafts But Not Cytokine Production In Vivo

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FTY720, a Novel Immunosuppressant, Induces Sequestration of Circulating Mature Lymphocytes by Acceleration of Lymphocyte Homing in Rats. II. FTY720 Prolongs Skin Allograft Survival by Decreasing T Cell Infiltration into Grafts But Not Cytokine Production In Vivo

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FTY720, a novel immunosuppressant, prolonged the survival of WKAH skin allografts transplanted into MHC-incompatible F344 rats. In this allograft model, the median survival time of the control group was 7 days, whereas those of the groups given FTY720 orally at 0.1 mg/kg and cyclosporin A (CsA) at 10 mg/kg were 10.5 and 11 days, respectively. In contrast, FTY720 (0.1 mg/kg) combined with CsA (10 mg/kg) synergistically prolonged allograft survival with a median survival time exceeding 70 days. To elucidate the mechanisms of this remarkable synergistic effect, mRNA expressions of IL-2 and IFN-γ and that of CD3 (δ-chain), which reflects T cell infiltration, in allografts were temporally analyzed using a semiquantitative PCR method. In WKAH skin allografts, mRNA levels of IL-2, IFN-γ, and CD3 were increased as compared with isograft controls, peaking on days 4 to 5. CsA (10 mg/kg) significantly inhibited elevations of IL-2 and IFN-γ mRNA, while slightly inhibiting that of CD3 mRNA in allografts. On the contrary, FTY720 (0.1 mg/kg) markedly inhibited the elevation of CD3 mRNA, while slightly inhibiting those of IL-2 and IFN-γ mRNA. FTY720 (0.1 mg/kg) combined with CsA (10 mg/kg) almost completely suppressed the intragraft expressions of mRNA for IL-2, IFN-γ, and CD3. Immunohistochemical staining and flow cytometric analysis also confirmed that FTY720 decreased T cell infiltration into allografts. From these results, the synergistic effect of FTY720 combined with CsA on prolongation of allograft survival is presumably based on the respective inhibitions of T cell infiltration and cytokine production in grafts. The Journal of Immunology, 1998, 160: 5493–5499.

Inbred strains of male F344 and WKAH rats were purchased from Charles River Japan (Atsugi, Kanagawa, Japan) and Japan SLC (Hamamatsu, Shizuoka, Japan), respectively. All rats were used at 4 to 6 wk of age.

Materials and Methods

Animals

Inbred strains of male F344 and WKAH rats were purchased from Charles River Japan (Atsugi, Kanagawa, Japan) and Japan SLC (Hamamatsu, Shizuoka, Japan), respectively. All rats were used at 4 to 6 wk of age.
Agents
FTY720 was synthesized according to previously described methods (1), dissolved in distilled water, and given orally. CsA (oral solution of Sandimmune, Sandoz, Basel, Switzerland) was dissolved in pure olive oil (Sigma Chemicals, St. Louis, MO) and administered orally. Control animals received the vehicle only.

Rat allogeneic MLC
Allogeneic MLC was performed using nylon nonadherent spleen cells from F344 rats as responder cells and mitomycin C (Kyowa Hakko, Tokyo, Japan)-pretreated spleen cells from WKHA rats as stimulator cells (5, 15). Responder cells at a concentration of 5 × 10⁵ cells/well were cocultured with equal numbers of stimulator cells in RPMI 1640 medium containing 5 μM 2-ME and 10% FCS (Boehringer Mannheim, Mannheim, Germany). After a 48-h culture at 37°C in 5% CO₂, the cells were recovered and used for RNA isolation.

Rat skin allograft
Full thickness skin grafts (square pieces 2.0 × 2.0 cm) were transplanted to the lateral thorax of recipient rats and covered with sterile bactericidal gauze. The entire chest was then wrapped with an elastic bandage. The dressings were removed on day 5 and the grafts were inspected daily until rejection, which was defined as more than 90% necrosis of the graft epithelium. The rat strain combination for MHC-incompatible transplantation was WKHA (RT1b) to F344 (RT1b/i) (3, 5). Either FTY720 or CsA was orally administered daily to the transplanted animals at the aforementioned doses.

mRNA analysis using RT-PCR
The skin grafts removed from the transplanted recipients or alloantigen-stimulated lymphocytes were homogenized with RNA isolation reagent (Nippon Gene, Tokyo, Japan). Total RNA was isolated according to the manufacturer’s protocol and then quantitated spectrophotometrically. Total RNA from the graft (1.2 μg) or from the lymphocytes (0.6 μg) was reverse transcribed in a 60-μl volume containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM MgCl₂, 1 mM each dNTP (dATP, dGTP, dTTP, and dCTP), 60 U RNase inhibitor (Takara, Kyoto, Japan), and 28 (CD3), or 24 (HPRT) U RNase inhibitor (Takara, Kyoto, Japan)-pretreated spleen cells from WKHA rats as stimulator cells (5, 15). Responder cells at a concentration of 5 × 10⁵ cells/well were cocultured with equal numbers of stimulator cells in RPMI 1640 medium containing 5 μM 2-ME and 10% FCS (Boehringer Mannheim, Mannheim, Germany). After a 48-h culture at 37°C in 5% CO₂, the cells were recovered and used for RNA isolation.

Semiquantitative PCR of target cDNA using IL-2-, IFN-γ-, CD3 (δ-chain)-, or HPRT-specific primers. Twofold dilutions of cDNA from rat skin allograft (WKHA donor to F344 recipient) 5 days after transplantation were amplified with IL-2-, IFN-γ-, CD3-, or HPRT-specific primers. A 10-μl aliquot was fractionated on 2% agarose gel and stained with SYBR Green I. Fluorescence intensities of the specific bands were visualized with a fluorescence image analyzer.

Immunohistochemical staining of skin allograft sections
Skin allografts were removed, embedded in tissue compound, and frozen in liquid N₂. Cryostat sections (6 μm thickness) were prepared, fixed with ice-cold acetone, and stained by immunoperoxidase methods. Briefly, sections were incubated with 5 mg/ml of biotinylated anti-rat CD3 mAb (clone: G4.18, PharMingen, La Jolla, CA) or biotin-conjugated anti-rat CD3 mAb (clone: biotin-conjugated G4.18, PharMingen, La Jolla, CA) and streptavidin-CyChrome (PharMingen). Numbers of CD3⁺ T cells in peripheral blood and skin allografts
Peripheral blood was periodically collected from a tail vein of allografted rats. The skin graft removed from transplanted recipients was chopped into small fragments, then incubated for 1 h at 37°C on a rocker in 4 ml of RPMI 1640 containing 10% FCS, 2 mg/ml collagenase (Calzyme, San Luis Obispo, CA), and 20 μg/ml DNase I (Boehringer Mannheim). The digested skin graft was passed through a stainless steel mesh, and skin parenchymal cells were sedimented over Ficoll buffer, density 1.090 g/cm³ (Immuno-Biologic Laboratories, Fujjoka, Japan), at 1700 × g for 20 min. The cells were stained with FITC-conjugated anti-rat CD3 mAb (clone: G4.18, Pharmingen, La Jolla, CA) and streptavidin-CyChrome (PharMingen). Numbers of CD3⁺ T cells were determined by flow cytometry with EPICS XL-MCL (Coulter, Miami, FL).

Results
Semiquantitative assessment of the measurement of IL-2, IFN-γ, and CD3 mRNA levels in allografts by the PCR method
For semiquantitative assessment of the measurement of IL-2, IFN-γ, and CD3 mRNA levels in allografts by the PCR method in our present study, a twofold dilution of cDNA solution prepared from allografts separated 5 days after transplantation (day 5) was amplified with IL-2-, IFN-γ-, CD3-, or HPRT-specific primers. The fluorescence intensity of the PCR product diminished as the cDNA level in the reaction mixture decreased (Fig. 1). Thus, the analysis by the PCR method was semiquantitative in this study.

Temporal analysis of IL-2, IFN-γ, and CD3 mRNA levels in rat skin allografts
Levels of IL-2, IFN-γ, and CD3 mRNA in allografts on days 2, 3, 4, and 5 were measured by the PCR method (Fig. 2). Isografts were analyzed in parallel to control for the nonspecific inflammatory response associated with the transplantation procedure. Control skin tissue (before transplant) contained detectable levels of IL-2, IFN-γ, and CD3 mRNA. The mRNA background levels of the isografts were only slightly enhanced. In contrast, IL-2, IFN-γ, and CD3 mRNA levels of allografts were markedly enhanced, peaking on day 4 (IL-2 and IFN-γ) and day 5 (CD3).

Effects of FTY720, CsA, and both on survival of rat skin allografts (WKHA) transplanted to MHC-incompatible rats (F344)
Figure 3 shows the effects of FTY720, CsA, and both on survival of rat skin allografts (WKHA) transplanted to MHC-incompatible rats (F344). In this allograft model, the median allograft survival was 7 days in vehicle-treated recipients (control group). The respective median graft survivals for FTY720-treated recipients at
doses of 0.1 mg/kg and 1 mg/kg were 10.5 and 21 days, respectively, while those of CsA-treated recipients at doses of 10 mg/kg and 30 mg/kg were 11 and 21 days, respectively. Thus, 0.1 mg/kg and 10 mg/kg were regarded as subtherapeutic doses for FTY720 and CsA, respectively. Combination treatment with FTY720 (0.1 mg/kg) and CsA (10 mg/kg) resulted in a remarkable prolongation, with median survival exceeding 70 days.

Figure 4 shows the time course change in the number of CD3+ T cells in the peripheral blood of allograft F344 rats. FTY720 (0.1 mg/kg) alone or combined with CsA (10 mg/kg) markedly decreased numbers of peripheral blood T cells during the administration period when compared with vehicle (control) or CsA (10 mg/kg) alone.

Effects of FTY720, CsA, and both on IL-2, IFN-γ, and CD3 mRNA levels in rat skin allografts

Figure 5 shows IL-2, IFN-γ, and CD3 mRNA levels in allografts of the recipients treated with FTY720 (0.1 mg/kg), CsA (10 mg/kg), and both agents on days 4, 5, 6, and 7. The mRNA levels in allografts of vehicle-treated recipients (control) on day 7 were not determined, as preparing the total RNA from rejected allografts was impossible. In the recipients treated with FTY720 (0.1 mg/kg), IL-2 and IFN-γ mRNA levels were enhanced in allografts and peaked on day 5, although the peak level was lower than that of vehicle-treated recipients. FTY720 (0.1 mg/kg) markedly suppressed the elevation of CD3 mRNA in the allograft until day 6, but failed to maintain this suppressive effect on day 7. On the contrary, CsA (10 mg/kg) markedly inhibited the elevations of IL-2 and IFN-γ mRNA in allografts, while having little effect on the CD3 mRNA level. In recipients treated with both FTY720 (0.1 mg/kg) and CsA (10 mg/kg), the elevations of IL-2, IFN-γ, and CD3 mRNA in allografts were markedly suppressed, and the levels were similar to those in isografts. This suppressive effect was maintained at least until day 14 (data not shown).

Figures 6 and 7 show the effects of subtherapeutic and therapeutic doses of FTY720 or CsA on IL-2, IFN-γ, and CD3 mRNA
levels in skin allografts 5 days after transplantation. A therapeutic dose of FTY720 (1 mg/kg) markedly suppressed the elevation of CD3 mRNA, while having little effect on the elevations of IL-2 and IFN-γ mRNA levels in the allograft. A subtherapeutic dose of FTY720 (0.1 mg/kg) had a similar effect. A subtherapeutic dose of CsA (10 mg/kg) did not, whereas a therapeutic dose of CsA (30 mg/kg) did, markedly inhibit the elevation of IL-2 and IFN-γ mRNA.

FTY720 did not affect mRNA expressions of IL-2 and IFN-γ in rat allogeneic MLR

CsA was reported to inhibit mRNA expression of Th1-associated cytokines, including IL-2 and IFN-γ, by Ag- or mitogen-stimulated Th cells (20, 21). The effects of FTY720 on alloantigen-induced mRNA expressions of IL-2 and IFN-γ were examined in comparison with those of CsA in allogeneic MLR using splenic T cells of F344 rats as responder cells and mitomycin C-pretreated WKAH rat spleen cells as stimulator cells (Fig. 8). CsA at 100 nM completely inhibited IL-2 and IFN-γ mRNA expressions in alloantigen-stimulated T cells. Unlike CsA, FTY720 up to 1000 nM affected neither IL-2 nor IFN-γ mRNA expression.

Effect of FTY720 on CD3+ T cell infiltration of rat skin allografts

Rat skin allografts were digested by collagenase on day 5, and then the number of CD3+ T cells in allograft were analyzed by flow cytometry.
cytometry with anti-rat CD3 mAb. As shown in Figure 9, FTY720 (0.1 mg/kg) markedly decreased CD3⁺ T cells in allografts. FTY720 (0.1 mg/kg) and CsA (10 mg/kg) were given orally on consecutive days following transplantation. Rat skin allografts were digested by collagenase on day 5, then numbers of CD3⁺ T cells in the allografts were analyzed by flow cytometry with anti-rat CD3 mAb. As a control, those of vehicle-treated recipients were also analyzed. Each column represents the mean ± SE of three animals.

Discussion
Patterns of cytokine expression in grafts of various transplantation models have been reported previously, and elevation of cytokine expressions in such grafts are believed to contribute to their rejection (9–14, 22, 23). These reports suggested that alloreactive T cells in allografts were activated by Th1-associated cytokines (IL-2 and IFN-γ) but not Th2-associated cytokines (IL-4 and IL-10), and that Th2-associated cytokines were essential for xenograft rejection. In addition to the elevation of cytokine expressions, T cell infiltration into the graft is thought to be an important component of graft rejection. Increased T cell infiltration of grafts has, in fact, been described in various graft models (10, 23). The expression of adhesion molecules related to T cell trafficking is enhanced in grafts, and mAbs of these molecules inhibit graft rejection (11, 24, 25). In our present MHC-incompatible allograft model, mRNA expressions of Th1-associated cytokines (IL-2 and IFN-γ) in the allografts were markedly enhanced (Fig. 2), whereas
that of a Th2-associated cytokine (IL-4) was undetectable (data not shown). In parallel, CD3 mRNA levels as a marker of T cell infiltration in the allografts were markedly enhanced (Fig. 2). T cell infiltration was also confirmed by immunohistochemical staining and flow cytometry with anti-CD3 mAb (Figs. 9 and 10). Therefore, infiltration of T cells into allografts and the activation of these T cells by Th1-associated cytokines presumably contributed to the allograft rejection observed in our present model.

In the current study, we determined the effects of FTY720 and CsA at high and low doses, to characterize these agents in detail (Figs. 6 and 7). The low dose of CsA (10 mg/kg) markedly inhibited the elevations of IL-2 and IFN-γ mRNA expressions in allografts, while having little effect on that of CD3 mRNA expressions (Figs. 5, 6, and 7). The ability for cytokine transcriptions by infiltrated T cells in FTY720-treated recipients may be higher than that in control recipients on day 5.

In the current study, we determined the effects of FTY720 and CsA at high and low doses, to characterize these agents in detail (Figs. 6 and 7). The low dose of CsA (10 mg/kg) markedly inhibited the elevations of IL-2 and IFN-γ mRNA expressions in allografts, while having little effect on that of CD3 mRNA expressions (Figs. 5, 6, and 7). The ability for cytokine transcriptions by infiltrated T cells in FTY720-treated recipients may be higher than that in control recipients on day 5.

References


