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A new immunosuppressant, FTY720, in canine kidney transplantation: effect of single-drug, induction and combination treatments

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Abstract Three different types of treatment were conducted to clarify the properties of a novel immunomodulator, FTY720, in canine kidney allograft models. Survival, biochemical and hematological tests, pharmacokinetics, and histopathology of grafts and extra-renal organs were analyzed. Accompanying a remarkable reduction in circulating lymphocytes, single-drug treatment of FTY720, ranging from 0.05 to 10 mg/kg, exhibited significant prolongation of graft survival without a dose-dependent effect. Short-course induction with FTY720 at 5 mg/kg per day exhibited similar anti-rejection effects as did single-drug treatment but no advantage in rescuing ongoing rejection. In combination with cyclosporine (CsA; 5 mg/kg) or tacrolimus (FK; 0.5 mg/kg), FTY720 had an additive effect. Trough blood concentrations of FTY720 were linearly correlated with dose. No animal showed critical adverse effects at any point. FTY720 holds promise as a candidate in a new category of drugs that can be combined with conventional agents for induction and maintenance immunosuppression in clinical organ transplantation.

Keywords FTY720 · Tacrolimus · Cyclosporine · Canine kidney transplantation · Single-drug treatment · Induction treatment · Combination treatment

Introduction

A novel immunosuppressive modulator, FTY720, is characterized by a reversible reduction of circulating peripheral blood lymphocyte (PBL) counts. This effect is thought to be caused by enhanced lymphocyte homing, at concentration orders of nanomoles per liter [1, 2, 3, 4], via augmented expression of adhesion molecules, G-protein-coupled chemokine receptors, and their ligands on the lymphocytes and/or high endothelial venules (HEVs) of lymph nodes (LNs) and Peyer's patches (PPs) [5, 6, 7, 8]. When FTY720 is administered at higher concentrations [i.e., at more than 2 or 4 μmol/l (700 or 1,400 ng/ml)], T cells from MRL/lpr mice, rat spleen cells, human peripheral blood cells, and/or other cell lines, including cancer cells, undergo apoptosis [3, 9, 10, 11, 12, 13, 14].

In experimental allogenic organ transplantation studies, FTY720 exhibited mild immunosuppression as a single-drug treatment and, when combined with conventional drugs, had synergistic immunomodulatory activity with or without adverse events [9, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25]. In recent phase II trials in denovo renal transplant recipients, FTY720 appeared effective in the prevention of acute rejection when given with cyclosporine (CsA) and steroids. Transient bradycardia was the most common adverse event in those trials [4, 26, 27].

Reported herein is our comprehensive study using canine allograft models to determine the effect of FTY720 as a single-drug treatment, as induction therapy, and in combination with CsA or tacrolimus (FK). Our goal was to obtain more practical information on the immunosuppressive potency, mechanism, pharmacokinetics, and toxicity of FTY720 for further application in clinical settings.

Materials and methods

Animals

The experiment was approved by the Animal Facility of the Hokkaido University and conducted in accordance with the guidelines proposed by the Institutional Animal Care and Use Committee. Adult female hybrids (11–13 kg) between American foxhounds, Labrador retrievers, and beagles were used as donors. Adult female beagles (9–11 kg) were used as recipients. After being starved overnight, the animals were anesthetized with thiopental sodium (25 mg/kg) for induction, and maintained with isoflurane (1.0%), nitrous oxide (2 l/min), and oxygen (2 l/min) by positive-pressure mechanical ventilation. Electrocardiogram, systolic and diastolic arterial pressures and esophageal temperature were monitored during surgery. Blood gases and electrolytes were measured at the following time points: after laparotomy, 10 min before graft reperfusion, 10 min after graft reperfusion, before abdominal closure, and before extubation. Abnormalities were corrected as necessary.

Operating procedure

Donors: Furosemide (1 mg/kg) and mannitol (300 mg/kg) were administered intravenously immediately after a midline laparotomy. Both kidneys were freed from the retroperitoneal wall, and the ureter and the renal vasculatures were skeletonized. After the abdominal aorta below the diaphragm had been clamped, 0.5 l of cold lactated Ringer's solution, containing 1,000 IUheparin sodium (Takeda Chemical Industries, Osaka, Japan), was flushed via a catheter that was inserted into the infra-renal abdominal aorta. The animals were hydrated with an infusion of 35 ml/kg per min of lactated Ringer's solution until renal grafts were removed.

Recipients: Kidney transplantation was performed by a standard intra-abdominal technique. Briefly, the kidney graft was transplanted into the right iliac fossa, anastomosing the renal artery to the proximal end of the transected common iliac artery, the renal vein to the side of the common iliac vein, and the ureter to the bladder. Bilateral recipient nephrectomy was immediately performed after reperfusion. Lactated Ringer's solution (25 ml/kg per h) was given during the operation. Furosemide (1 mg/kg) and mannitol (300 mg/kg) were administered intravenously immediately before reperfusion of the graft. All animals were allowed to eat and drink from the following morning. Cefazolin, 1 g (Fujisawa Pharmaceutical Co., Osaka, Japan), was given intravenously during the operation and continued for 3 days postoperatively.

Immunosuppressive drugs

FTY720, kindly supplied by the MitsubishiWellfaid Pharmaceutical Co. (Osaka, Japan), was dissolved in distilled water. CsA (oral solution of Sandimmune; Novartis, Basle, Switzerland) was dissolved in pure olive oil (Sigma Chemical Co., St. Louis, Mo., USA). FK, supplied as a powder (Fujisawa Pharmaceutical Co), was suspended in normal saline solution. All agents were given orally via a catheter inserted into the pharyngo-esophagus. The doses of these agents were adjusted for body weight, which was monitored every morning. Doses had been established in a previous study of canine liver transplantation [22].

Experimental groups

Single-drug treatment: The animals were randomly classified into six groups ($n = 6$ each) to identify the dose-dependent effects of FTY720. Doses per day were 0.05 mg/kg (group 2), 0.1 mg/kg (group 3), 1 mg/kg (group 4), 5 mg/kg (group 5), and 10 mg/kg (group 6). Animals with no treatment (group 1) were used as a control. Treatment was started on the morning after the operation and continued until the animals died or were euthanized according to the following criteria: serum creatinine (Cr) > 1,000 μmol/l, blood urea nitrogen (BUN) > 25,000 μmol/l, body weight loss > 30% of the initial weight, severe weakness, or survival > 90 days.

Induction treatment: The induction study consisted of four groups ($n = 6$ each). FTY720 at 5 mg/kg per day was given on days −3, −2, and −1, prior to operation (group 7), on days 0, 1, and 2 after operation (group 8), on days 3, 4, and 5 after operation (group 9), or on day 0 prior to operation (group 10).

Combined treatment: Combined treatment groups ($n = 6$ each) and doses per day were as follows: group 11 (CsA, 5 mg/kg), group 12 (FTY720, 0.1 mg/kg, and CsA, 5 mg/kg), group 13 (FTY720, 5 mg/kg, and CsA, 5 mg/kg), group 14 (FK, 0.5 mg/kg), group 15 (FTY720, 0.1 mg/kg, and FK, 0.5 mg/kg) and group 16 (FTY720, 5 mg/kg, and, FK, 0.5 mg/kg). Treatment was given orally from the first postoperative day until the 90th day after the operation, unless the animals were euthanized under the criteria used for the single-drug groups.

Biochemical and hematological studies. Blood samples were collected from the peripheral vein on days 0, 1, 3, 5, 7, 10 and 14 after the operation and twice a week thereafter. Serum levels of Cr, BUN, aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin (TB), and blood glucose (BG) were measured by an autoanalyzer (type 7020, Hitachi Medico, Tokyo, Japan). Hematological analyses were performed with an autoanalyser (Coulter A^C T10; Coulter Corporation, Miami, Fla., USA) immediately after we had collected the blood to determine hematocrit, hemoglobin, and red blood cell (RBC), white blood cell (WBC) and platelet (Plt) counts. WBC analyses were performed with slides after cells had been stained by the Giemsa method.

Drug blood levels: Using whole-blood samples stored at −20°C, we measured FTY720 concentrations by gas chromatography–mass spectrometry [22]. Blood concentrations of CsA and FK were determined by radioimmunoassay and enzyme immunoassay, respectively.

Tissue FTY720 concentration: At postmortem examinations, tissues from the kidney, mesenteric lymph node, spleen, and thymus were collected and stored at −80°C. The FTY720 concentration in these tissues was measured as previously described [22].

Histological study: Complete postmortem analyses were performed. Renal allografts and extra-renal organs were fixed with formalin, paraffin embedded, and stained with hematoxylin–eosin (HE). The severity of graft rejection was classified as follows: none, no rejection; mild, interstitial mononuclear cell infiltration greater than 25% of the parenchyma; moderate, with intimal arteritis; and severe, with transmural arteritis, arterial fibrinoid change, and/or necrosis of medial smooth muscle cell.

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) was utilized to detect DNA fragmentations in apoptotic cells in the kidney, mesenteric lymph node, spleen, and thymus. The degree of apoptosis was scored in accordance with subjective scales, from 0 to 3. All studies were performed by one pathologist with no knowledge of groups or treatments.

Statistical analysis

Data were expressed as the mean and standard deviation. Animal survivals were expressed as the median number of days. Comparison among groups was performed with the log–rank test. Interim group analysis was performed by Student's *t*-test. *P* values less than 0.05 were considered statistically significant. The analyses were carried out with the statistical program Stat View (version 4.5; Abacus Concepts, Berkeley, Calif., USA).

Results

Animal survival

A total of 108 renal transplantations was performed. Twelve animals (13.6%) that died from vascular thrombosis ($n=4$), intussusception ($n=4$), peritonitis ($n=2$), bleeding ($n=1$) or pneumothorax ($n=1$) during the immediate postoperative period were excluded from the analysis. Animal survivals are summarized in Table 1.

Single drug treatment: The median survival time of untreated control animals was 12 days. All of the treated groups showed statistically significant but modest prolongation of survival compared to control. Although group 3 animals, given 0.1 mg/kg per day of FTY720, lived slightly longer than animals in the other groups (groups 2 and 4), no apparent dose-dependent prolongation of survival was observed. Three of the six animals given 10 mg/kg per day of FTY720 developed moderate-to-severe gastrointestinal bleeding.

Induction treatment: Compared with that in the control, FTY720 induction was effective in prolonging animal survival when it was given for 3 days before (group 7) or after operation (group 8), and even when it was given once on the day of operation (group 10). Delayed treatment starting on day 3 after operation (group 9) had no effect. No adverse events were seen in any of the induction treatment groups.

Combination treatment: Animals treated with CsA at 5 mg/kg per day (group 11) or FK at 0.5 mg/kg per day (group 14) survived for median periods of 22 and 29 days, respectively. Co-administration of FTY720 at 0.1 mg/kg per day (group 12) or 5 mg/kg per day (group 13) with 5 mg/kg per day of CsA resulted in a slight, but not statistically significant, prolongation of survival when compared with each single-drug treatment group (group 3, 5 or 11). When each dose of FTY720 was combined with 0.5 mg/kg per day of FK, the median survival time of groups 15 and 16 was prolonged to 49.5 days and 50 days, respectively. One animal in group 16 lived for 90 days. Among the animals treated with a combination of FTY720 and FK, weakness was the main reason for euthanasia.

Biochemical studies

Graft function: Changes of serum Cr levels are shown in Fig. 1. In untreated animals, the mean serum Cr began to rise after post-operative day (POD) 5 and was greater than 1,000 μmol/l after POD 10. In single-drug treatment animals, the onset of Cr elevation was delayed for several days (to POD 14 in group 3 and to POD 10 in the other groups), but renal dysfunction with high levels of Cr, 1,000 μmol/l, and BUN, 25,000 μmol/l, in these

Table 1 Effect on canine kidney allografts of 720 FTY single-drug treatment, induction, and combination therapy with CsA or FK

Group	Number in group	Dose (mg/kg per day)			Graft survival	Median survival time
		FTY	CsA	FK	(days)	(days)[d]
Control						
1	6	–	–	–	10, 10, 10, 14, 14, 14	12
Single-drug treatment						
2	6	0.05	–	–	17, 17, 17, 21, 21, 21	19[1]
3	6	0.1	–	–	21,21, 22, 23[b], 27, 38[a]	22.5[1,2,4]
4	6	1	–	–	13[a], 13, 15, 15, 18, 19	15[1]
5	6	5	–	–	15, 17, 19, 21, 24, 33[b]	20[1,4]
6	6	10	–	–	14[a], 14, 15[a], 25[a], 26, 29[b]	20[1]
Induction treatment (timing)						
7 (days −3, −2, −1)	6	5	–	–	14, 15, 21, 21, 21, 22	21[1]
8 (days 0, 1, 2)	6	5	–	–	13, 13, 18, 21, 22, 28	19.5[1]
9 (days 3, 4, 5)	6	5	–	–	10, 12, 13, 17, 17, 22	15
10 (day 0)	6	5	–	–	18, 19, 20, 24, 24, 24	22[1]
Combination treatment						
11	6	–	5	–	15, 17, 22, 22, 24, 47[b]	22[1]
12	6	0.1	5	–	18, 21, 24, 27, 29, 29	25.5[1]
13	6	5	5	–	21, 23, 24, 30, 40, 53	27[1]
14	6	–	–	0.5	14, 19, 24, 34, 40[c], 44	29[1]
15	6	0.1	–	0.5	20, 21, 49[c], 50, 57[c], 57[c]	49.5[1,14]
16	6	5	–	0.5	23, 38, 43[c], 57[c], 86[c], >90	50[1,5]

[a]Animal developed gastrointestinal bleeding
[b]Animal was euthanized due to severe emaciation (body weight loss of 30%)
[c]Animal was euthanized due to weakness
[d]The group demonstrated a significant difference ($P < 0.05$) in graft survival, by log-rank test

animals was similar to that in control animals. Cr levels in most of the animals that were given induction treatment remained below 500 μmol/l until POD 10, but all of the grafts were finally rejected, with Cr levels exceeding 1,000 μmol/l. In animals given the combination of FTY720 and FK, Cr levels remained below 500 μmol/l until the animals died or were euthanized, while the animals treated with FTY720 and CsA had Cr levels close to 1,000 μmol/l at the final sampling points.

Extra-renal organ function: Beyond 3 days after kidney transplantation, there were no abnormalities in ALT, AST, LDH, TB or BG levels in untreated or treated animals.

Hematological studies

Red blood cells, white blood cells and platelets: RBC and platelet counts in all the experimental animals were stable during the observation period. The mean WBC count in recipient animals was 9,075 ± 2,261/μl preoperatively, rose by twofold to threefold at the first postoperative week, and remained at the higher level thereafter in most of the treated groups.

WBC analysis: Preoperative values were 68.1 ± 6.6% (6,273 ± 2,004/μl) of neutrophils, 28.8 ± 5.7% (2,529 ± 377/μl) of lymphocytes, 2 ± 1% (187 ± 92/μl) of eosinophils, 1 ± 0.6% (92 ± 46/μl) of monocytes and no basophils. Neutrophils accounted exclusively for the increase in total WBC count in all groups, while basophils, eosinophils and monocytes showed no important changes. In animals given no treatment or only CsA (group 11) or FK (group 14), the number of lymphocytes remained basically unchanged. In animals given FTY720, however, lymphocytes were markedly reduced, to less than 30% of initial level at POD 3 and less than 10% throughout the observation period (Fig. 2).

Pharmacological studies

Blood concentration of FTY720, CsA and FK: The serum trough levels of FTY720, CsA and FK are shown in Figs. 3 and 4. FTY720 blood levels increased in a dose-dependent fashion. At POD 7, they were 1.64 ± 0.7 ng/ml at 0.05 mg/kg per day, 6.29 ± 4.3 ng/ml at 0.1 mg/kg per day, 21.0 ± 11.8 ng/ml at 1 mg/kg per day, 178.5 ± 62.8 ng/ml at 5 mg/kg per day, and 203.0 ± 68.2 ng/ml at 10 mg/kg per day (Fig. 3).

When FTY720 was combined with CsA or FK, the blood concentration of FTY720 did not significantly

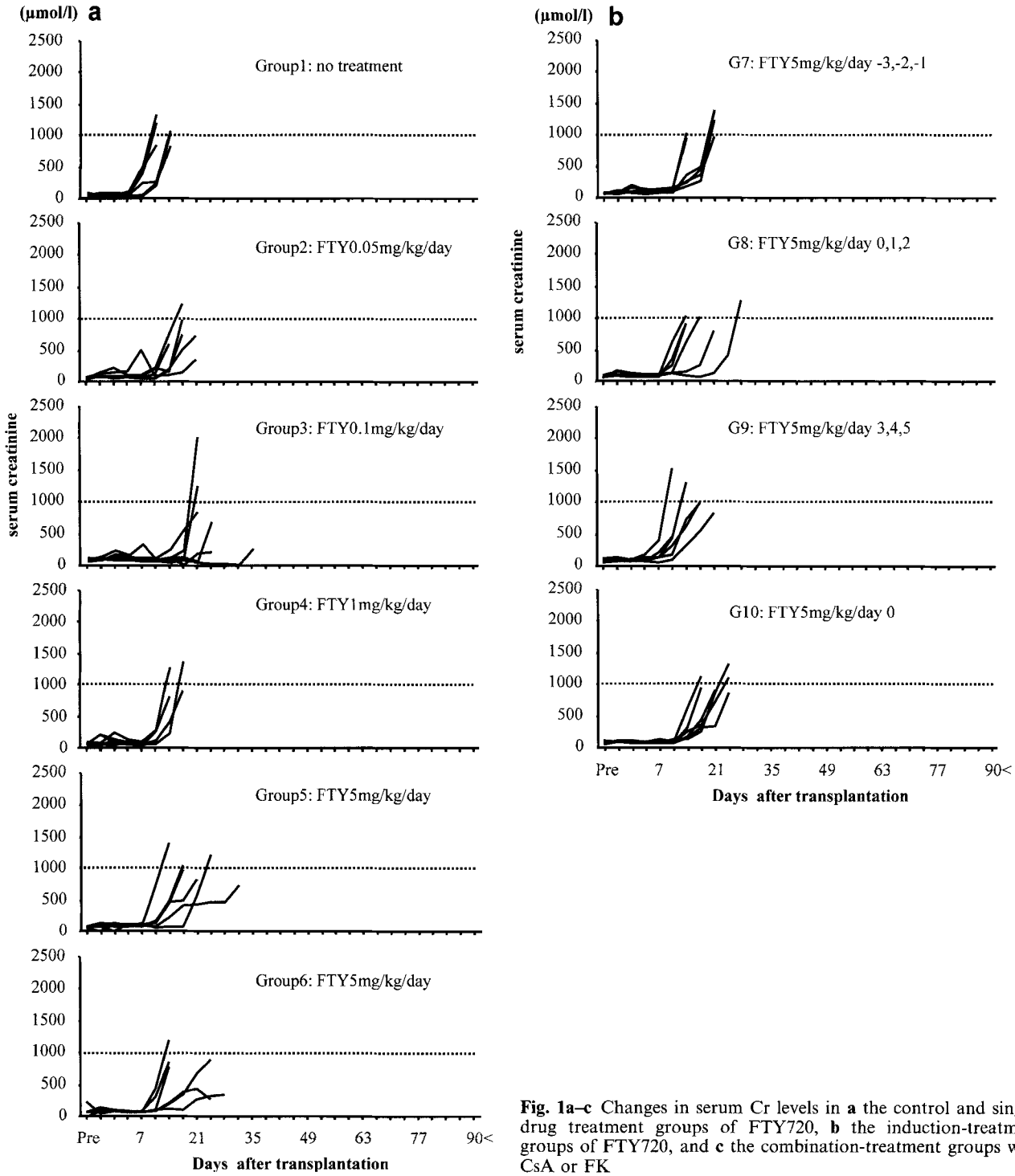

Fig. 1a–c Changes in serum Cr levels in **a** the control and single-drug treatment groups of FTY720, **b** the induction-treatment groups of FTY720, and **c** the combination-treatment groups with CsA or FK

differ from that with single-drug treatment (Fig. 4). FTY720 at daily doses of 0.1 mg/kg and 5 mg/kg had no influence on the trough levels of CsA and FK. CsA levels remained at 20 to 240 ng/ml, and FK levels were between 0.5 and 9.6 ng/ml, in each of the three combination-treatment groups.

Tissue concentration of FTY720: FTY720 concentrations in the graft, mesenteric lymph nodes, spleen and thymus in groups 2 and 4 are shown in Fig. 5. Compared with blood concentrations, tissue concentrations of FTY720 were 100-times higher, particularly in the spleen. In group 2, the tissue concentration of FTY720

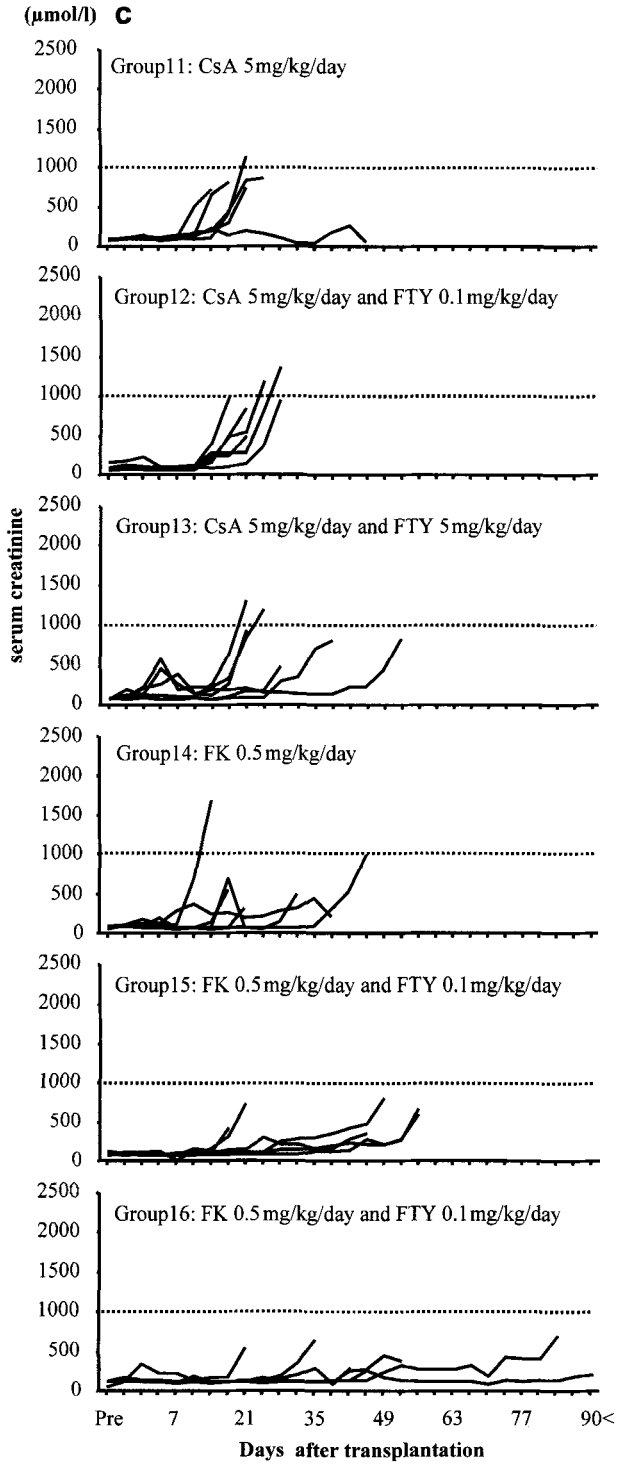

Fig. 1a–c (Contd.)

in the spleen was significantly higher than that in the kidney graft and thymus. Drug level in the spleen was 528.9 ± 143.7 ng/ml at 0.05 mg/kg per day and 6,614.0 ± 3,126.2 ng/ml at 1 mg/kg per day, respectively.

Histopathological studies

Renal allografts: Severe rejection, indicated by transmural arteritis, arterial fibrinoid change, and/or necrosis of medial smooth muscle cells, was observed in grafts from untreated animals. Findings were essentially similar in the grafts from animals given single-drug or induction treatment, although the degree was slightly less in the group 6 animals. The animals given FTY720 and CsA had moderate-to-severe rejection, while animals given FTY720 and FK had only moderate rejection.

Extra-renal organs: Abnormal changes in extra-renal organs are listed in Table 2. The heart, liver and pancreas showed no important structural changes, except for the focal fibrinoid degeneration of cardiac vasculatures in three animals (one each in groups 1, 2 and 4). Regardless of the FTY720 dose, most of the treated animals had focal fibrosis and congestion around alveoli and bronchioles. Compared with the control, all animals given FTY720 by single-drug, induction, or combination treatment had augmented lymphocyte infiltrates at the mucosa and submucosa layers of the small intestine. In particular, lymphocyte accumulation was most prominent at the ileum, and there was universal expansion of lymphofollicles at the Peyer's patches. TUNEL staining showed sporadic apoptotic cells in the spleen, mesenteric lymph nodes and thymus in both untreated and FTY720-treated animals.

Discussion

The present study in a canine kidney transplantation model has shown that FTY720 exhibits modest immunosuppression as a single-drug treatment at various dosages ranging from 0.05 mg/kg per day to 10 mg/kg per day, that graft survival time after briefperioperative induction therapy is similar to survival time after single-drug treatment, and finally, that CsA and FK each have an additive effect.

Regardless of the dose and the type of treatment, FTY720 administration is accompanied by a profound reduction of circulating lymphocyte counts in the peripheral vein. Gastrointestinal (GI) toxicity and morphological abnormalities in the lung, focal fibrosis, and congestion around alveoli and bronchioles were important findings in the animals exposed to FTY720. Co-administration of FTY720 had no influence on the drug levels of CsA or FK. From histopathological analyses, accelerated lymphocyte homing, particularly to the Peyer's patches at the ileum, appeared to be the mode of action by this agent.

In general, the higher the dose administered, the greater the immunosuppressive effect obtained with conventional anti-rejection drugs. In rat models of skin,

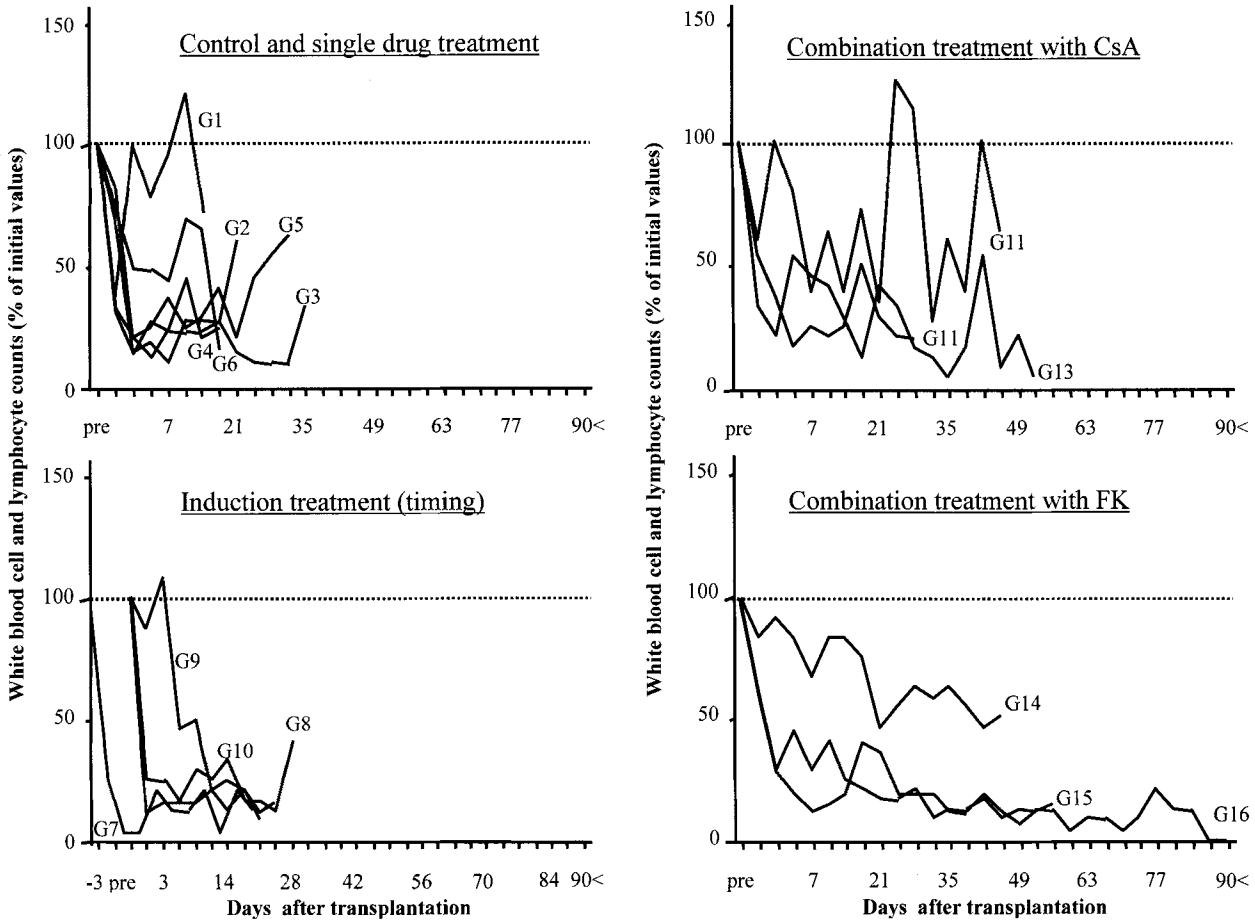

Fig. 2 Lymphocyte counts in the control and single-drug treatment groups, induction treatment, and combination treatment with CsA or FK expressed as a mean percentage of initial values (*G* group)

heart, liver and small intestine transplantation, FTY720 showed dose-dependent prolongation of graft survival [8, 9, 15, 16, 21, 23]. In those experiments, therapeutic doses were between 2 and 10 mg/kg per day.

In contrast, our study, using this agent, failed to demonstrate a dose-dependent effect on canine kidney graft survival, even though the dose was varied from 0.05–10.0 mg/kg per day. Among these groups, animals treated with FTY720 at 0.1 mg/kg per day exhibited the longest graft survival periods accompanied by stable lymphocyte reduction (Fig. 2). Dose-dependent effects of FTY720 on this model might exist with an oral dose of less than 0.1 mg/kg per day, because of significantly different animal survival times in-between the doses of 0.05 and 0.1 mg/kg per day. In addition, animals treated with a high dose of FTY720, 10 mg/kg per day, had a strong likelihood of GI toxicity. Study of the single-drug treatment concluded that the therapeutic dose of FTY720 might be very low, and only the reduction of PBL counts was insufficient to prevent the canine kidney allograft rejection completely. These findings suggest that, in a clinical setting, it may be unnecessary for the drug dose to be escalated beyond that which achieves profound reduction of lymphocyte counts. Indeed, in the clinical trials, combined use of FTY720 at a dose of 2 or 2.5 mg/day reduced the lymphocyte counts to 30% of the pretransplant level, with a significant decrease in acute rejection episodes, whereas a dose of 5 mg q.d. showed no important benefits but caused adverse side effects, such as bradycardia.

For induction treatment in the present study, we selected the higher dose of FTY720 at 5 mg/kg per day [28]. A 3-day course starting before or on the day of transplantation prolonged animal survival similarly to continuous single-drug treatment but with no evident side effects.

Although the rescue by FTY720 of on-going rejection of rat skin, heart and liver allografts has been reported [29, 30, 31], in our study the agent was not effective when it was started 3 days after transplantation. More importantly, however, FTY720 was effective as an induction therapy even if it was given only once on the day of transplantation. Immunosuppressive efficacy by preoperative administration of this agent has been

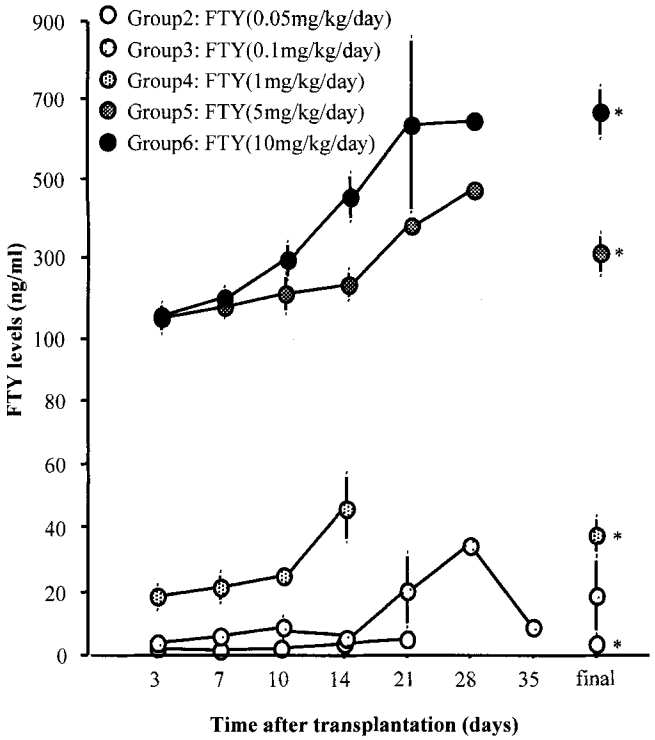

Fig. 3 FTY720 trough blood concentrations in recipients treated with FTY alone. Values are expressed as mean ± SD. In each group the statistical significance between FTY levels on POD 3 and the final day was calculated by Student's unpaired *t*-test (*$P < 0.05$)

confirmed by others in rat heart, liver, and islet transplantation [17, 18].

In combination with conventional drugs (i.e., CsA, FK and RAD), FTY720 has been shown to exhibit additive or synergistic effects on various allografts in small animals [15, 16, 21, 23, 25]. In our previous study using a canine liver transplantation model [22], FTY720 at 0.1 mg/kg per day with 5 mg/kg per day of CsA prolonged median animal survival times from 40 days (CsA alone) to 74 days, while the same dose of FTY720 with 0.5 mg/kg per day of FK reduced the survival from 83.5 days (FK alone) to 30.5 days due to over-immunosuppression. In the present study, we tried to determine whether increasing the daily dose of FTY720 from 0.1 mg/kg to 5 mg/kg without altering the CsA or FK dose would improve graft function and animal survival. We found no difference between the lower and higher dose of FTY720, however. Our observation is consistent with that of Troncoso et al [20], who demonstrated that in combination with sub-therapeutic doses of CsA given to achieve serum trough concentrations of 40 to 200 ng/ml, intravenous administration of FTY720 at 0.1 mg/kg per day or 0.3 mg/kg per day yields a similar prolongation of graft survival, 71 days versus 63 days, while the combination of 1 mg/kg per day of FTY720 and CsA reduced graft survival to 48 days by over-immunosuppression. Those findings suggest that, as found in single-drug treatment, a minimal dose of FTY720 is sufficient to produce maximum immunosuppression in a combination therapy.

In the groups treated with combined FTY720 and FK (groups 15 and 16), weakness and suffering, with persistence of moderate uremia, was the main reason for euthanasia. In addition, CsA in combination with FTY720 could not show an additive effect. The cyclosporine used in this study was Sandimmune, the older, less potent version. A better effect on potency might have been obtained with Neoral.

FTY720 induces profound reduction of peripheral lymphocyte counts, as found by many studies, including ours [22, 28]. Chiba et al. and Brinkmann et al. reported that the precise mechanisms of PBL reduction by FTY720, at concentration orders of nanomoles per liter, have been considered to be accelerated lymphocyte homing/trafficking to lymphoid tissues [5, 6, 7, 8]. Suzuki and others, in in vitro studies with rat spleen cells, human peripheral blood cells, and cancer cell lines, have found that, at a serum concentration of 2 μmol/l (700 ng/ml) or more, FTY720 induces apoptotic cell death resulting in PBL reduction [9, 10, 11, 12, 13, 14]. In the present study, FTY720 at a low dose of 0.05 mg/kg per day induced significant depletion of lymphocyte counts in the peripheral blood, accompanying definite expansion of the lymphofollicles at the Peyer's patches. Blood and tissue drug concentrations of the LNs at this dose were less than 4 ng/ml and 400 ng/ml, respectively; these concentrations were not enough to induce apoptosis in the vitro study. Even when the dose was increased to 1 mg/kg per day, TUNEL staining showed no increase of apoptotic cells in lymphoid tissues at drug concentration of 4,000 ng/ml. This drug level did induce apoptosis in the vitro study. Thus, our findings suggest that FTY720 exhibits its precise action by accelerating lymphocyte trafficking and not apoptosis.

The time to maximum plasma concentration (T_{max}) and half-life ($T_{1/2}$) after oral administration of FTY720 is 8 h to 9 h and 12 h to 29 h in rats and dogs, respectively [32]. In baboons, T_{max} is 2 h to 24 h and $T_{1/2}$ is 24 h to 48 h [1]. In human trials, T_{max} and $T_{1/2}$ were 12 h to 36 h and 89 h to 157 h, respectively [3]. In our study, FTY720 blood trough levels were correlated with oral dosages in all groups. However, the final trough level was significantly increased compared with that on POD 3 in each group, especially in animals given higher doses (Fig. 3). In addition, tissue concentrations of the agent were approximately 100-times higher than blood levels (Fig. 5). There have been no reports focused on the tissue accumulation of FTY720 in large animals. These observations might suggest that FTY720 must be used very cautiously, given its long half-life in humans. Trough blood concentrations at a therapeutic dosage of 0.1 mg/kg per day in dogs ranged from 5 to 10 ng/ml.

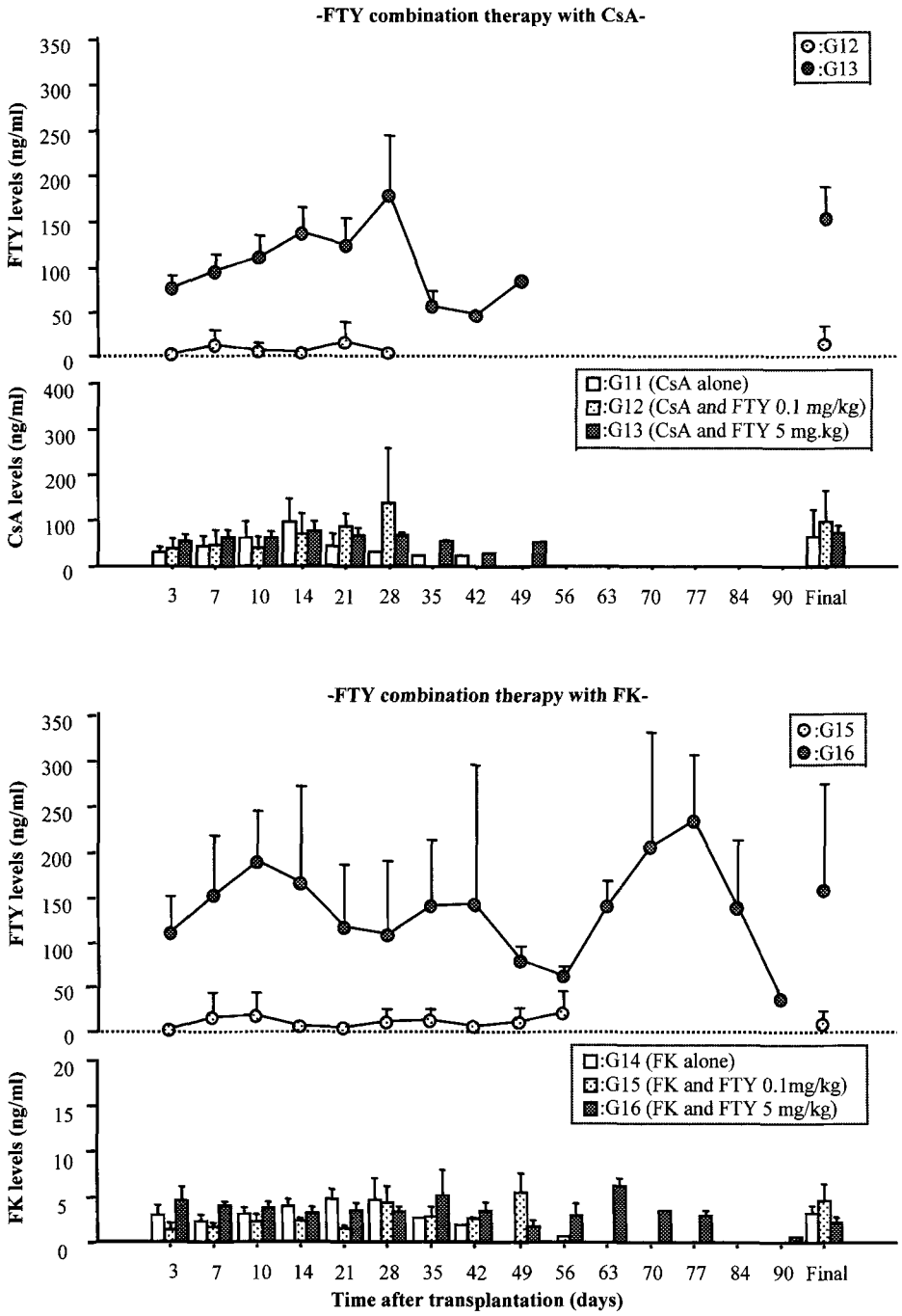

Fig. 4 FTY720, CsA, and FK trough blood concentrations in recipients given combination therapy. Values are expressed as mean ± SD. In each group, there was no significant difference between FTY, CsA, or FK levels on POD 3 or the final day (*G* group)

These target trough levels were similar to those with the recommended trough levels, ranging from 1.25 to 5.27 ng/ml in monkeys [20] and from 0.97 to 8.76 ng/ml in humans [27].

There were no drug interactions between FTY720 and CsA or FK in this study. This result was consistent with that of others [9, 22]. In human trials, we might propose that trough blood levels of FTY720 should be adjusted to a minimum concentration, probably at some nanogram per milliliter level chosen on the basis of data on the graft survival and degree of lymphocytes reduction and pharmacological profiles in this study, to allow for the maximum PBL reduction.

Six animals treated with FTY720 exhibited GI bleeding (one in group 3, one in group 4, and three in group 6). This may suggest that at 10 mg/kg per day, FTY720 may provoke gastrointestinal toxicity in dogs. No liver toxicity or hyperglycemia and agranulocytosis were observed in the biochemical and hematological analyses. In the extra-renal organ examination, the

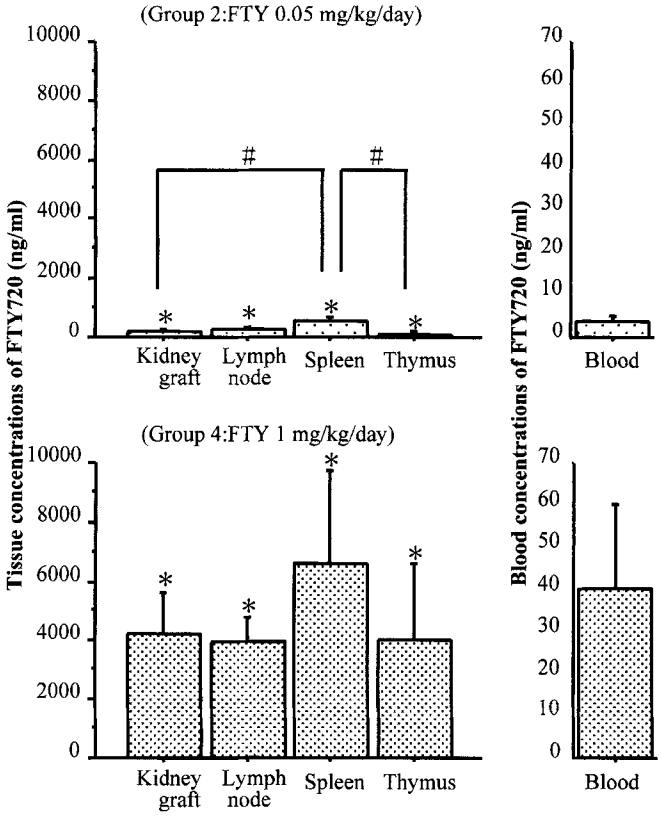

Fig. 5 *Columnar graph on the left side* shows the FTY720 concentrations in host tissues (kidney graft, lymph node, spleen, and thymus) that were exposed to FTY at doses of 0.05 and 1 mg/kg ($n=3$). In each group, final blood concentrations of FTY are shown in the *columnar graph on the right side*. Values are expressed as mean ± SD. $*P<0.05$ compared with FTY blood concentration in each group, $\#P<0.05$ between the different tissues

lung exposed to FTY720 showed bronchitis or pneumonia with infiltration of macrophages into alveoli;

Table 2 Histopathological abnormalities in extra-renal organs of animals on FTY720 monotherapy

Group	FTY doses (mg/kg per day)	Heart: fibrinoid degeneration	Lung: fibrosis	Liver: congestion	Ileum: proliferation of lymph follicles
1	None	1/6	0/6	3/6	0/6
2	0.05	1/6	1/6	4/6	3/6
3	0.1	0/6	0/6	4/6	5/6
4	1	1/6	6/6	4/6	5/6
5	5	0/6	4/6	6/6	3/6
6	10	0/6	2/6	6/6	4/6

these distinctive changes are obvious side effects of FTY720 in this model. Congestive changes in the liver, which were observed in control animals as well, were attributed to animal weakness due to uremia following kidney grafts rejection. Finally, there was no infectious event in any animal given single-drug or combination treatment. In the clinical trials with FTY720, lung effects and GI toxicity have not been observed, and the most common adverse events were transient bradycardia and headache [4]. Overall, no serious adverse events were reported in any pre-clinical or clinical study.

In conclusion, FTY720 will most likely have a practical role as an adjuvant immunosuppressant, in a novel category of optional immunomodulators, that would make it possible to reduce the adverse effects of conventional immunosuppressant drugs in clinical organ transplantation.

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