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Protective effects of FTY720 on chronic allograft nephropathy by reducing late lymphocytic infiltration

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Background. Lymphocytic infiltration is obvious throughout early and late stages of chronic allograft nephropathy. Early infiltrating lymphocytes are involved in initial insults to kidney allografts, but the contribution of late infiltration to long-term allograft attrition is still controversial. Early application of FTY720 reduced the number of graft infiltrating lymphocytes, and inhibited acute rejection. The present study investigated the potential of FTY720 to reduce the number of infiltrating lymphocytes even at a late stage, and, thus, slow the pace of chronic allograft nephropathy.

Methods. Fisher (F344) rat kidneys were orthotopically transplanted into Lewis recipients with an initial 10-day course of cyclosporine A (1.5 mg/kg/day). FTY720, at a dose of 0.5 mg/kg/day, or vehicle was administered to recipients either from weeks 12 to 24 or from 20 to 24 after transplantation. Animals were harvested 24 weeks after transplantation for histologic, immunohistologic, and molecular analysis.

Results. FTY720, either initiated at 12 or 20 weeks after transplantation, reduced urinary protein excretion, and significantly ameliorated glomerulosclerosis, interstitial fibrosis, tubular atrophy, and intimal proliferation of graft arteries at 24 weeks after transplantation. Furthermore FTY720 markedly suppressed lymphocyte infiltration and decreased mRNA levels of interleukin-10 (IL-10), transforming growth factor-β (TGF-β), and platelet-derived growth factor-B (PDGF-B) but enhanced the number of apoptotic cells in grafts.

Conclusions. FTY720 ameliorated chronic allograft nephropathy even at advanced stages. Furthermore, our data suggest that this effect was achieved by a reduction of graft-infiltrating lymphocytes.

Thanks to substantial improvements in short-term outcome, kidney transplantation has become the treatment of choice for most patients with end-stage renal diseases (ESRD). However, the rate of long-term graft failure due to chronic allograft nephropathy remained at 50% to 80%. [1]. Alloantigen-dependent as well as nonalloantigen-dependent factors are involved in the development of the process. Most of these factors induce the recruitment of lymphocytes into the grafts [2, 3]. Clinically, biopsies of renal allografts have demonstrated a predominance of T lymphocytes, not only in grafts undergoing acute but also chronic allograft nephropathy ($35 \pm 10\%$ of area infiltrate) [4, 5]. Similarly, in a well-established rat model of chronic allograft nephropathy, lymphocyte infiltration patterns in allografts remained stable throughout different periods after transplantation, and were consistently more substantial than those noted in isografts [6, 7].

Lymphocytes infiltrating graft tissue early after transplantation, in concert with the cytokines they release, contribute to early insults to renal allografts, and may result in acute rejection or predispose for chronic allograft deterioration [8, 9]. However, it remained controversial over the past decade whether lymphocytic infiltration late after transplantation plays an essential role. Some studies have indicated that in chronic allograft nephropathy lymphocytes are important mediators of a persistent alloantigen dependent immune response [10, 11] and the development of arteriosclerosis during the ongoing process of chronic allograft nephropathy [12, 13]. However, others argue that late lymphocytic infiltration may serve only as an adjunct of chronic rejection, and the simple presence of T cells during this period does not necessarily indicate active destruction [14–16], as severe T-cell infiltration can be observed in either chronically rejecting or stable allografts [17].

Recently, a new immunosuppressive agent, FTY720, which is a synthetic analogue of a natural compound from the fungus *Isaria sinclairii* has been developed [18]. Chemical structure and mode of action differ from conventional immunosuppressants [19]. FTY720 prolonged the survival of skin [20, 21], heart [22], liver [23, 24], kidney [25–27], small bowel [28], pancreaticoduodenal [29], and islet allografts [30], and prevented the development of graft-versus-host immune responses [31] in rats and

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canines. Moreover, FTY720 even stopped ongoing acute rejection, in heart and liver allografts [23, 32]. Combined treatment with FTY720 and other immunosuppressive agents, such as cyclosporine A [33] and tacrolimus (FK 506) [34, 35], exerted synergistic effects, and lead to permanent acceptance of allografts in some cases. Although the exact mechanisms of the immunosuppressant have not been fully elucidated, FTY720 induces apoptosis of peripheral blood and graft infiltrating lymphocytes [23], and accelerates the trafficking of circulating lymphocytes to lymphoid tissues [36]. As a result, the number of infiltrating lymphocytes in graft tissues is markedly reduced, and their detrimental effects on acutely rejecting allografts are thus diminished.

Reports concerning effects of FTY720 on ongoing chronic allograft nephropathy do not exist. Therefore, in the present study, we initiated FTY720 treatment in a well-established rat model of chronic allograft nephropathy at week 12 after transplantation, when lymphocytic influx into allografts becomes apparent [6, 7], and at week 20, when allograft fibrogenesis is ongoing [37].

METHODS

Animals

Naive inbred male Fisher (F344, RT1v1) and male Lewis (RT1) rats (Charles River, Sulzfeld, Germany), weighing 200 to 250 g, were kept under standard conditions and fed with rat chow and water ad libitum. All experiments were approved by a governmental committee on animal welfare and followed the "Principles of Laboratory Animal Care" (NIH publication No. 86–23, revised 1985).

Kidney transplantation

Under ketamine (Ketamin) (100 mg/kg intraperitoneally) (CP-Pharma, Burgdorf, Germany) and xylacine (Rompun) (10 mg/kg intraperitoneally) (Bayer, Leverkusen, Germany) anesthesia the left donor kidney was removed, cooled, and positioned orthotopically into the recipient. Donor and recipient renal artery, vein, and ureter were anastomosed end-to-end with 10-0 Prolene sutures. No ureteral stent was used. To overcome infectious complications rats received cephtriaxone (Rocephin) (20 mg/kg/day, intramuscularly) (Hoffmann-La Roche AG, Grenzach-Wyhlen, Germany) on the first postoperative day. Animals were treated with low-dose cyclosporine A (1.5 mg/kg/day subcutaneously) (Novartis GmbH, Nürnberg, Germany) over the first 10 days after transplantation to overcome an initial episode of acute rejection. The contralateral native kidney was removed on the tenth postoperative day.

Experimental design

FTY720 (Novartis Pharmaceutical Industries, Basle, Switzerland) was dissolved in physiologic saline, and administered to recipients by oral gavage at 0.5 mg/kg.

Transplanted animals were assigned to four experimental groups ($N = 8$/group) treated either with FTY720 from week 12 to week 24 (FTY 12-24) or from week 20 to week 24 (FTY 20-24), or vehicle from week 12 to 24 (vehicle 12-24) or from week 20 to 24 (vehicle 20-24) after transplantation. Kidney allografts were harvested 24 weeks after transplantation.

Functional measurements

Every 4 weeks, body weight was measured and 24-hour urine samples were collected using metabolic cages with a urine cooling system. Quantitative urine protein was nephelometrically determined. Serum and urine creatinine levels were measured and creatinine clearance was calculated at the end of the study.

Histology

For histology, kidney tissues were fixed in 4% buffered formalin, embedded in paraffin, and stained with either hematoxylin and eosin or periodic acid-Schiff (PAS). Glomerulosclerosis was defined as a collapse of capillaries, adhesion of the obsolescent segment of Bowman's capsule, and entrapment of hyaline in the mesangium [38]. All glomueruli in each section were counted and the proportion of sclerosed to total glomeruli was expressed as percentage. Chronic allograft nephropathy in terms of glomerulopathy, tubular atrophy, interstitial fibrosis, and vascular intimal proliferation were quantified according to the Banff '97 classification [38] and graded as follows: 0, no signs of chronic allograft nephropathy; grade 1, mild chronic allograft nephropathy with mild fibrosis and tubular atrophy; grade 2, moderate chronic allograft nephropathy with moderate fibrosis and tubular atrophy; and grade 3, severe chronic allograft nephropathy with severe fibrosis and tubular atrophy.

Immunohistochemistry

For immunohistology, cryostat sections (4 μm) were fixed in acetone, air dried, and stained individually with primary monoclonal mouse–derived antibodies against monocytes/macrophages (ED1) and CD5+ T lymphocytes (OX19) (Serotec Camon Labor-Service GmbH, Wiesbaden, Germany). After incubation with the primary antibody, sections were incubated with rabbit antimouse IgG and thereafter the alkaline phosphatase antialkaline phosphatase (APAAP) complex (Dako A/S, Hamburg, Germany). Cells staining positive were counted and expressed as cells per field of view. At least 20 fields of view per section and per specimen were

evaluated at 400× magnification by two independent observers in a blinded fashion.

TUNEL assay

Terminal deoxynucleotidyl transferase (dTd)-mediated deoxyuridine triphosphate (dUTP) nick end-labeling (TUNEL) assay was performed on frozen sections fixed in paraformaldehyde 4% as described elsewhere [39]. Briefly, sections were incubated with Triton 0.1% and sodium citrate 0.1% at 4°C. After washing they were incubated with TUNEL solution. Sections were washed with stop/wash buffer followed by washing and incubation with a rabbit antidigoxygenin antibody. Antibody binding was visualized using fast red chromogen solution. Positive controls were treated with DNase I and processed as described above. Negative controls were incubated with phosphate-buffered saline (PBS) instead of TUNEL solution. Sections were counterstained with hematoxylin. All positive tubular epithelial cells in each section were counted and related to the number of fields of view per section.

RNase protection assay

Total RNA was extracted using Trizol reagent (Gibco, Karlsruhe, Germany) according to the manufacturer's instructions. Intragraft mRNA expression specific for interleukin (IL)-1α, IL-1β, IL-2, IL-5, IL-6, IL-10, interferon-γ (INF-γ), tumor necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β1), platelet-derived growth factor-B (PDGF-B), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Riboquant Multi-Probe template set) (Pharmingen, Becton Dickinson GmbH, Hamburg, Germany) was determined by RNase protection assay using the In vitro Transcription Kit and RPA Kit (Pharmingen, Hamburg, Germany) as previously described [40]. Briefly, ^{32}P-labeled antisense riboprobes were synthesized with the use of T7 RNA polymerase transcription in the presence of [α^{32}P]-UTP. Radiolabeled antisense riboprobes were then hybridized with 10 μg of total RNA extracted from cultured cells at 56°C overnight. After hybridization, RNase A + T1 were added to digest unhybridized RNA and duplex RNA hybrids were separated by electrophoresis on a 5% polyacrylamide gel. Intensities of the protected bands were quantified by a phosphorimaging analyzer (Fuji-BAS 1500) (Düsseldorf, Germany) and the ratios of the investigated genes to GAPDH (internal control) were calculated.

Statistical analysis

Data are presented as mean ± SEM. Parametric data were compared using one-way analysis of variance (ANOVA), followed by multiple pairwise comparison according to the Newman-Keuls test. Nonparametric data were tested using the Kruskal-Wallis one-way analysis of ranks. Discrete data were compared using chi-square test. A P value of less than 0.05 was considered significant.

RESULTS

In comparison to controls, treatment with FTY720, initiated from either week 12 or week 20 after transplantation, significantly ameliorated signs of chronic allograft nephropathy (Fig. 1A to D) in terms of glomerulosclerosis, interstitial fibrosis, tubular atrophy, and intimal proliferation of graft arteries at the end of the follow-up period.

The percentage of sclerotic glomeruli was markedly reduced in recipients treated with FTY720 from week 12 (20.0 ± 2.1% vs. 32.2 ± 2.5% in controls) ($P < 0.01$) (Fig. 2). More interestingly, even FTY720 treatment from week 20 reduced glomerulosclerosis ($P < 0.05$) (Fig. 2), although to a lesser extent than in those treated from week 12 ($P < 0.05$) (Fig. 2). Similarly, FTY720 resulted in a reduced degree of glomerulopathy independent of the treatment period. Most glomeruli in FTY720-treated animals were of normal size with only minor hypercellularity and patent capillary loops, while those in controls revealed a more pronounced hypertrophy, with focal and segmental proliferation.

Along with ameliorated glomerular injury, arteries appeared relatively normal in FTY720-treated animals, with only focal ballooning of endothelial cells or with vascularization of the media (Fig. 1D). In addition, only minor, nonspecific tubular changes were observed in grafts of treated animals, and interstitial fibrosis was weak. By contrast, sections of allografts from controls demonstrated more severe interstitial fibrosis, tubular atrophy, and intimal proliferation of the arteries. Some cortical arteries contained mild intimal proliferation (<20% of vessel luminal cross-section), while some had more than 50% of luminal occlusion due to neointimal expansion (Fig. 1C). Thus, animals from both treatment groups developed a lower Banff score as compared to controls ($P < 0.05$) (Fig. 3). Although animals treated with FTY720 from week 12 tended to have less severe tubular and vascular changes than those treated from week 20, the difference did not reach statistical significance (Fig. 3).

Twenty weeks after transplantation, urinary protein excretion was lower in animals treated with FTY720 from week 12 than in controls (23.8 ± 2.4 mg/dL/24 hours vs. 29.0 ± 4.8 mg/dL/24 hours ($P > 0.05$) (Fig. 4A). The difference became significant at week 24 (27.2 ± 3.7 mg/dL/24 hours vs. 42.5 ± 6.0 mg/dL/24 hours ($P < 0.05$) (Fig. 4A). On the other hand, in animals treated with FTY720 from week 20 urinary protein excretion at week 24 was only slightly lower than in controls (31.9 ± 6.1 mg/dL/24 hours vs. 41.0 ± 5.9 mg/dL/24 hours) (Fig. 4B). Serum creatinine levels and creatinine clearance did not differ significantly

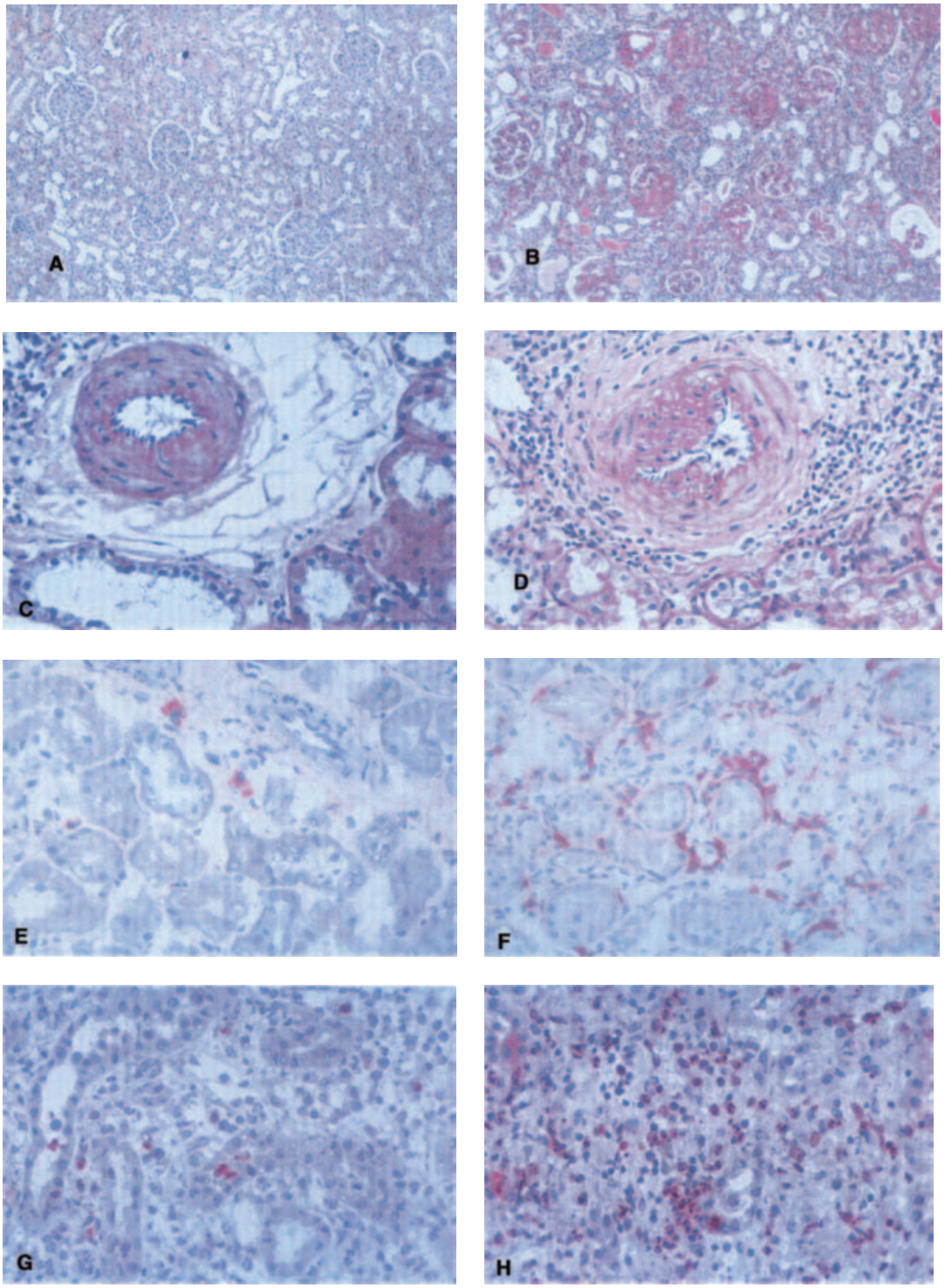

Fig. 1. Microphotos of periodic acid-Schiff (PAS) staining (*A* to *D*), immunohistochemical staining with anti-CD5 monoclonal antibody (*E* and *F*), and terminal deoxynucleotidyl transferase (dTd)-mediated deoxyuridine triphosphate (dUTP) nick end-labeling (TUNEL) staining (*G* and *H*) in renal allografts harvested at 24 posttransplantation week. (A) FTY720 treatment resulted in minor changes. (B) Vehicle treatment resulted in severe deterioration of graft tissue. (C) Normal artery after FTY720 treatment. (D) Intima proliferation after vehicle treatment. (E) Slight lymphocyte infiltration in FTY720-treated animals. (F) Intense lymphocyte infiltration in vehicle-treated animals. (G) Diffused distribution of TUNEL-positive infiltrates in FTY720-treated animals. (H) Limited amount of TUNEL-positive infiltrates in vehicle-treated animals.

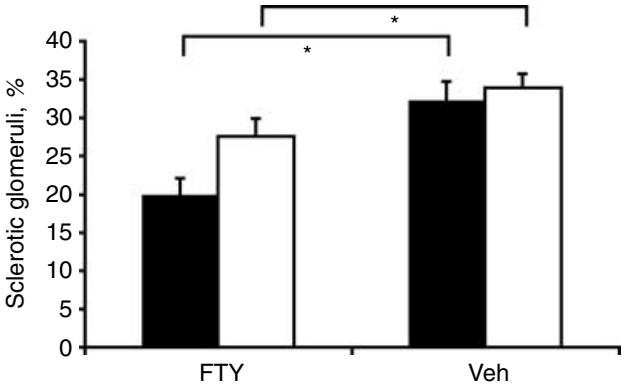

Fig. 2. Percentage of sclerotic glomeruli in renal allografts treated with FTY720 (FTY) or vehicle (Veh) from 12 to 24 (■) or from 20 to 24 (□) post-transplantational weeks. $^*P < 0.05$.

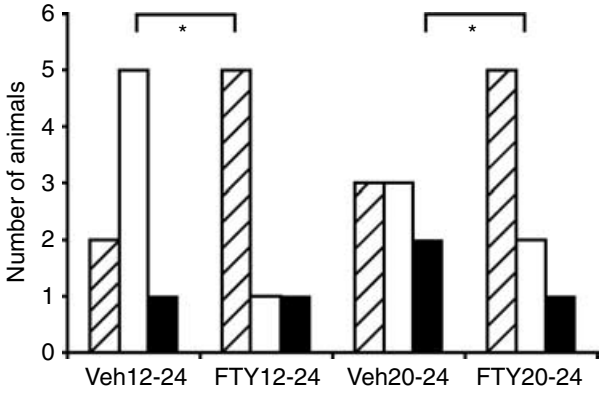

Fig. 3. Banff classification of renal allografts demonstrating mild (grade I, striated columns), moderate (grade II, □), and severe (grade III, ■) chronic allograft nephropathy. $^*P < 0.05$ as compared to vehicle-treated animals using chi-square test.

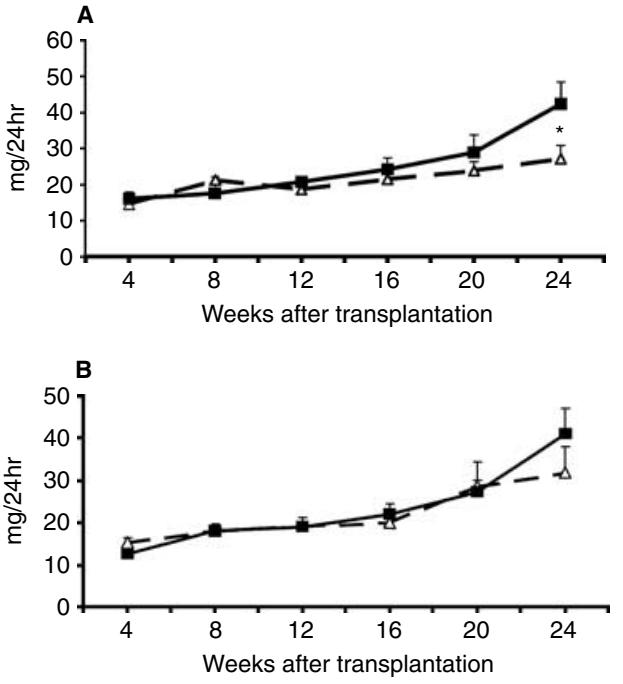

Fig. 4. Changes in 24-hour urinary protein excretion during the course of the experiment in animals treated with FTY720 (■) and vehicle (△) from 12-24 (*A*) and from 20-24 (*B*) weeks after transplantation. $^*P <$ 0.05 as compared to controls at the same time point.

between the groups at the end of the follow-up (creatinine clearance 0.36 ± 0.05 mL/min/100 g body weight in vehicle 12-20 vs. 0.37 ± 0.04 mL/min/100 g body weight in FTY 12-24) ($P > 0.05$), and 0.37 ± 0.05 mL/min/100 g body weight in vehicle 20-24 vs. 0.38 ± 0.06 mL/min/100 g body weight in FTY 20-24) ($P > 0.05$).

To investigate whether FTY720 hindered the development of chronic allograft nephropathy by reducing mononuclear infiltrates in graft tissues, we determined the intensity of T-cell and monocyte/macrophage infiltration with immunohistochemistry for CD5 and ED1, respectively. FTY720 treatment reduced the intensity of lymphocytic infiltration in renal allgrafts. While grafts of controls were diffusely infiltrated by CD5-positive lymphocytes in interstitial areas, spreading from perivascular to periglomerular regions and even surrounding Bowman's capsules (Fig. 1E), grafts from FTY720-treated animals had a relatively circumscript infiltration pattern with a significantly lower number of T lymphocytes (Fig. 5A) ($P < 0.05$) distributed primarily around vessels (Fig. 1F), without any significant differences between animals treated for a longer period (week 12-24) or shorter period (week 20-24) (Fig. 5A) ($P > 0.05$). On the other hand, monocyte/macrophage infiltration in renal allografts was not influenced by FTY720 treatment, as ED1-positive cell counts were similar in all groups (Fig. 5B).

In order to explore whether the reduced lymphocyte infiltrates were accompanied by enhanced apoptosis, we evaluated in situ DNA fragmentation in graft infiltrates with the TUNEL assay. Apoptotic cells were identified basing on TUNEL staining and morphologic signs of apoptosis, including chromatin condensation, detachment of cytoplasm from the environment, and formation of apoptotic bodies (Fig. 1G and H).

Apoptosis affected both the infiltrating leukocytes and tubular epithelial cells. To no surprise, FTY720 treatment increased the number of apoptotic graft infiltrating lymphocytes as compared to controls. While only ~4% of infiltrating lymphocytes were undergoing apoptosis in vehicle-treated animals, FTY increased the percentage to around 8% ($P < 0.05$) (Fig. 6). However, longer treatment did not further enhance apoptosis in graft infiltrates, as no significant differences were observed between animals treated with FTY720 from week 12 or from week 20 ($P > 0.05$) (Fig. 6). On the other hand, we observed no difference regarding the number of apoptotic tubular epithelial cells between treatment groups and controls (data not shown).

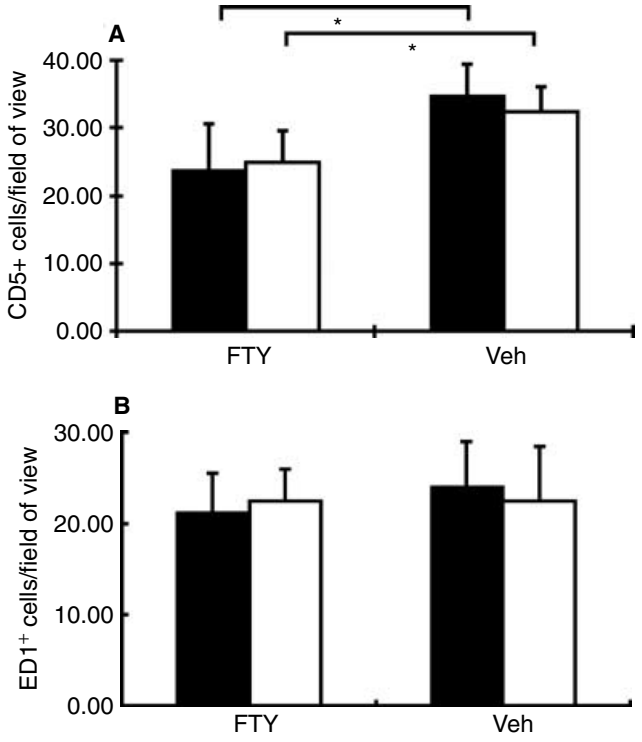

Fig. 5. Immunohistochemical evaluation of CD5-positive (A) and ED1-positive (B) cellular infiltration in renal allografts treated with FTY720 (FTY) or vehicle (Veh) from 12-24 (■) and from 20-24 (□) posttransplantational weeks. *$P < 0.05$ vs. vehicle-treated animals.

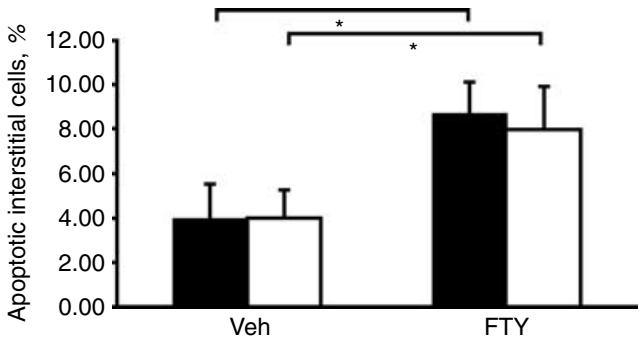

Fig. 6. Percentage of apoptotic infiltrating cells in renal allografts treated with FTY720 (FTY) or vehicle (Veh) from 12-24 (■) or from 20-24 (□) posttransplantational weeks. *$P < 0.05$ as compared to vehicle-treated animals.

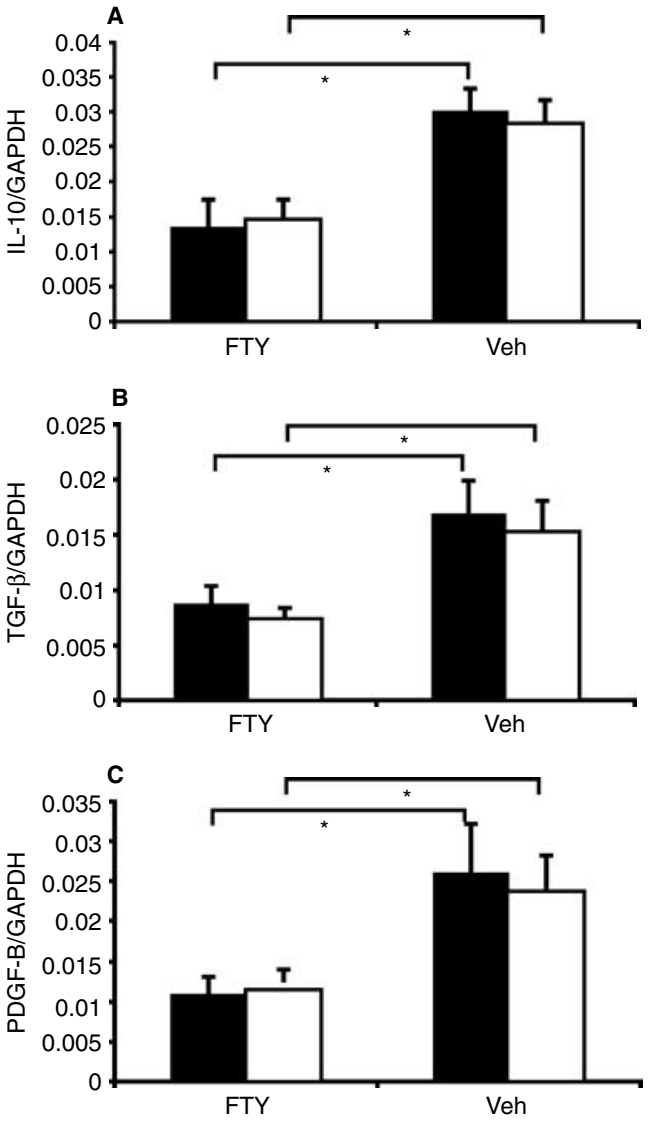

Fig. 7. RNase protection assay analysis of interleukin (IL)-10 (*A*), transforming growth factor-β (TGF-β) (*B*), and platelet-derived growth factor-B (PDGF-B) (*C*) mRNA expression in renal allografts treated with FTY720 (FTY) or vehicle (Veh) from 12-24 (■) and from 20-24 (□) posttransplantational weeks. *$P < 0.05$ vs. vehicle-treated animals.

We further investigated whether a reduction of lymphocytic infiltrates resulted in a decreased expression of cytokines and growth factors. As shown in Figure 7, FTY720 treatment from weeks 12 and 20 reduced IL-10 mRNA expression in allografts by twofold and 2.5-fold, respectively, as compared to controls ($P < 0.01$). Additionally, TGF-β mRNA and PDGF-B mRNA expression were significantly higher in controls than in FTY720-treated animals ($P < 0.05$) (Fig. 7). No difference was observed in IL-10, TGF-β, and PDGF mRNA expression between the two FTY720 treatment groups. Furthermore, the expression of IL-1α, IL-1β, IL-2, IL-5, IL-6, IFN-γ, and TNF-α mRNA in renal grafts did not differ between treatment and control groups (data not shown).

No differences in body weight were observed between FTY720-treated animals and controls during the course of the experiment (body weight at the time of harvesting 476 ± 28 g in vehicle12-24 vs. 463 ± 19 g in FTY 12-24 ($P > 0.05$) and 462 ± 21 g in vehicle 20-24 vs. 449 ± 23 g in FTY 20-24 ($P > 0.05$). Likewise, mean arterial blood pressure of FTY720-treated animals did not significantly differ from controls [72.4 ± 6.2 mm Hg in vehicle 12-24 vs. 77.8 ± 6.8 in FTY 12-24 ($P > 0.05$) and 72.9 ± 4.7 in vehicle 20-24 vs. 78.9 ± 6.6 in FTY 20-24 ($P > 0.05$).

DISCUSSION

It has been well documented that lymphocytic infiltration is prominent in the interstitial areas of transplanted kidneys during chronic allograft nephropathy [8, 9]. In our previous studies, we observed that improved long-term renal graft outcome was usually accompanied by a reduction of infiltrating lymphocytes [41]. However, whether the infiltration of T lymphocytes was cause or consequence of chronic allograft nephropathy remained unclear. In the present study, we demonstrated that reduction of lymphocytic infiltrates in rat renal allografts at late stages after transplantation attenuated the progression of chronic allograft nephropathy.

Allograft biopsies of patients undergoing chronic rejection demonstrated lymphocytic infiltration of perivascular as well as fibrotic interstitial areas, and even within atophic tubules [4, 5, 42, 43]. In agreement, we observed massive CD5-positive T cells infiltrating the interstitial areas of renal allografts in the control animals of our present model. Presently, FTY720 emerges as the only immunosuppressant that exerts its effects on acute rejection by reducing lymphocytic infiltration. Short-term application of the drug markedly reduced lymphocytic infiltration in rat cardiac, liver, skin, and renal allografts undergoing acute rejection [20–26]. Combined treatment with FTY720 and cyclosporine A in these models even depleted T-cell infiltrates and completely suppressed the intragraft expressions of T-cell–associated cytokines [33–35]. Our present results extended these findings to a model of chronic rejection, as late application of FTY720 initiated, either when lymphocytic migration into allografts became obvious (week 12), or when renal fibrosis was ongoing (week 20), significantly inhibited the number of infiltrating lymphocytes at late stages after transplantation.

Despite the presence of T lymphocytes in chronic rejection, it is still under controversy whether these cells are responsible for chronic graft deterioration. In contrast to acute rejection, T-cell–associated cytokines, such as IL-2, IL-4, and IFN-γ, were not up-regulated at late stages after transplantation, and the expression of these cytokines did not correlate to long-term allograft dysfunction [44, 45]. Additionally, intense lymphocytic infiltration could also be observed in stable allografts long after transplantation [17].

In this context, our present study aimed to give insights into the contribution of infiltrating T lymphocytes present late after renal transplantation to chronic allograft nephropathy by diminishing the infiltration with FTY720. Along with the marked reduction of infiltrating lymphocytes, herein, long-term renal allograft function assessed by 24-hour urinary protein excretion was improved, and fibrogenesis of the allografts was remarkably alleviated as a lower percentage of sclerotic glomeruli and less severe interstitial fibrosis and arteriosclerosis were observed in FTY720-treated recipients. In agreement, evidence has been given in vitro that T cells isolated from engrafted renal tissues from patients with chronic allograft nephropathy were sensitized by peptides derived from allogenic major histocompatibility complex [46] and were highly response to phytohemagglutinin (PHA) and Conconvalin A activation [47]. Collectively, these data suggested that infiltrating T lymphocytes present at late posttransplantational stages participate in chronic insults to renal allografts.

More interestingly, although the same extent of lymphocytic infiltration was observed at the end of the follow-up period in both FTY720 treatment groups, we found a better long-term outcome in allograft function and pathologic changes in animals with FTY720 treatment before allograft fibrogenesis was obvious (week 12). This probably indicated that not only infiltrating T lymphocytes present during excessive allograft fibrogenesis (20 weeks after transplantation), but also those present prior to obvious renal fibrosis (from 12 to 20 posttransplantational week) were involved in the ensuing graft damage, and removing these infiltrates in both periods helped to further reduce the pace of chronic allograft nephropathy.

The above findings suggested that T cells infiltrating renal allografts late after transplantation were activated, rather than in-activated or resting lymphocytes. Indeed, following the reduction of lymphocytic infiltration, the mRNA expression of IL-10, a Th2-related cytokine, was markedly decreased despite the stable low expression of Th1-type cytokines, such as IL-2 and IFN-γ. These data suggest that infiltrating lymphocytes present late after transplantation were activated and were probably Th2 related. Th2-type cytokines have been associated with the development of chronic rejection in allografts surviving long-term [48]. In addition, IL-10 may promote the proliferation of fibroblasts, and, thus, fibrogenesis and artheriosclerosis in renal allografts [49]. Recently, it was shown that supernatants of infiltrating lymphocytes isolated from chronically rejecting renal allografts enhanced PDGF-induced proliferation of vascular smooth muscle cells [46]. Hence, we deduced that infiltrating lymphocytes at late posttransplantational stages might release Th2-type cytokines, including IL-10, which contributed to long-term renal allograft attrition. Late application of FTY720 eliminated Th2 cytokines probably by reducing lymphocytic infiltration, and thus limited the progression of chronic allograft nephropathy.

In addition to reduced IL-10 levels, intragraft mRNA expression of TGF-β and PDGF-B was also significantly lower in FTY720-treated recipients. PDGF is likely to be a critical event in fibroblast proliferation in vitro [50] and in vivo [51] and has been associated with the development of chronic allograft nephropathy [52]. The concomitant reduction of intragraft expression of these growth factors by FTY720 also lent support to the antifibrogenic effects of the drug on renal allografts.

Collectively, we deduced that late FTY720 treatment protected renal allografts from chronic allograft nephropathy by eliminating lymphocytic infiltrates in graft tissues. Hence, the question arose what was responsible for the reduction of lymphocytic infiltrates in our model. We further found that this effect was associated with enhanced apoptosis of the graft infiltrates in FTY720-treated animals. In agreement, FTY720 induced apoptosis of infiltrating lymphocytes in renal and liver allografts during acute rejection [23, 53]. This was supported by previous in vitro results demonstrating a number of apoptotic events in FTY-treated lymphocytes, including genomic DNA fragmentation, chromatin condensation, and formation of apoptotic bodies [54]. Furthermore, FTY720 led lymphocytes to programmed cell death through intracellular activation of common signal pathways for apoptosis, including the activation of the caspase cascade and protein kinases, by induction of permeability transition to release cytochrome c from mitochondria [55].

On the other hand, the binding capacity of lymphocytes to allografts also determined the number of the infiltrates in graft tissue. Our previous study suggested that lymphocyte binding in the allografts rises at 8 weeks, peaks at 12 weeks and decreases at 16 weeks after transplantation in this model. Such binding is determined partly by the affinity of lymphocytes with graft tissues, which is related to the expression of adhesive molecules in allografts, and partly by the affinity of lymphocytes with lymph nodes and Peyer's patches due to the expression of integrins on lymphocytes. It has been suggested that treatment with FTY720 may increase the expression, affinity, or avidity of α_4-integrin (VLA-4) expression based upon the effect of preincubation of treated lymphocytes with an anti-VLA-4 monoclonal antibody to prevent the lymphodepletion effect of FTY720. If FTY720 alters VLA-4 expression, it would "home" lymphocytes to high endothelial venules in lymph nodes and Peyer's patches and, thus, away from the allografts [56]. This may be responsible for the reduction of lymphocyte infiltration due to a decrease in lymphocytic influx into allografts. In this context, a longer application of FTY720 may result in further decrease in the intensity of lymphocytic infiltration. However, our results showed that the reduction of lymphocyte infiltration was independent of the FTY720 treatment period, implying that infiltrate reduction may mainly be a result of FTY720-induced apoptosis. Studies on *aly/aly* mice that lack lymph nodes and Peyer's patches support our findings by showing that FTY720 administration in these mice suppressed immune functions in the same manner as in normal mice by inducing lymphocyte apoptosis [57]. However, further studies are needed to evaluate the alteration of lymphocyte trafficking in chronic allograft nephropathy.

CONCLUSION

Our data suggest that lymphocytic infiltration at even late stages after transplantation contribute to the development of chronic allograft nephropathy. Therapies directed toward the reduction of infiltrating lymphocytes, such as FTY720-induced apoptosis, ameriolate long-term renal allograft deterioration and thus reduce the pace of chronic allograft nephropathy.

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