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FTY 720A mediates reduction of lymphocyte counts in human renal allograft recipients by an apoptosis-independent mechanism

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Abstract The novel immunosuppressive compound FTY 720A posseses a mode of action which is different from all other immunosuppressive drugs. The most prominent feature is a reversible decrease in peripheral lymphocyte counts observed in animal experiments. We investigated in the first human trial (phase 1) whether FTY 720A induces apoptosis of peripheral blood mononuclear cells (PBMC) in stable renal allograft recipients. Monitoring of lymphocyte counts revealed a significant and dose-dependent decrease within 6 h post-FTY 720A dose: placebo 5.1 %; 0.25 mg 36.4 %; 0.5 mg 40.8 %; 0.75 mg 39.4 %; 1 mg 45.8 %; 2 mg 67.2 %; 3.5 mg 64.9 %. PBMC apoptosis rates did not change, as determined before intake of FTY 720A and 2 h, 6 h, 24 h and 96 h post-FTY 720A dose. We detected no significant difference in apoptosis rates between patients who received placebo or FTY 720A. However, in vitro experiments showed that high concentrations of FTY 720 A induced apoptosis in human PBMC.

Key words FTY 720A · Transplantation · Immunosuppression · Lymphopenia · Apoptosis

Introduction

FTY 720A, a synthetic derivative of the sphingosine myocine (ISP-1), is a novel immunosuppressive agent [5]. In several animal models, FTY 720A (0.5–1 mg/kg per day) orally significantly prolonged graft survival. It has been shown in these animal models that FTY 720A acts synergistically with cyclosporine A (CyA) [2]. The metabolism of FTY 720 is cytochrome P450 independent. Therefore combined therapy with FTY 720A with (cytochrome P450-dependent) CyA may be a promising option. FTY 720A exerts a unique mechanism of action compared to all other known immunosuppressive compounds. FTY 720A did not inhibit lymphocyte proliferation and did not interfere with IL-2 synthesis or Il-2 dependent signal transduction [3]. Recently, Kiuchi et al. reported that the prochiral hydroxymethyl groups at the quaternary carbon were important on immunosuppressive activity of FTY 720A [4]. So far a cellular target molecule of FTY 720A has not been elucidated. Except for its potent immunosuppressive action, the mechanism of action of FTY 720A is still poorly understood. In animal studies, FTY 720A induced reversible lymphopenia [2, 5]. The reduction of lymphocytes seems to be linked to the immunosuppressive effects of FTY 720A. There is an ongoing discussion as to whether this lymphopenia is caused by apoptosis [5] or by altered lymphocyte homing [3]. Here, we investigated whether FTY 720 A induces apoptosis in human peripheral blood mononuclear cells in human renal allograft recipients.

Materials and methods

Study design

We performed a double-blind, ascending single oral dose (0.25–3.5 mg), phase I study with 16 stable renal allograft recipients. The 16 subjects in this study were at least 12 months post-transplant

with therapeutic levels of CyA and methylprednisolone and a maximal serum creatinine level of 3.0 mg/dl.

Determination of lymphocyte counts

Blood samples were withdrawn before intake of FTY 720A/ placebo and 2 h, 6 h, 10 h, 24 h and 96 h post-FTY 720A/placebo dose. Lymphocyte counts were determined by routine laboratory diagnostics using an automatic cell counter.

Isolation of peripheral blood lymphocyte counts (PBMC)

PBMC were isolated by Ficoll density centrifugation as reported previously [1]. An aliquot of 10 ml citrate coagulated blood was diluted with 10 ml phosphate buffered saline (PBS). After centrifugation, PBMC were withdrawn from the interface between Ficoll and plasma. PBMC were washed with PBS and resuspended (10^6 PBMC/ml) in binding buffer (Pharmingen).

Determination of cell viability

Cell viability was determined by the MTT-test as described previously FCS [1]. Samples of 10^6 PBMC/ml were cultured in RPMI-1640 medium supplemented with 10% and penicillin/streptomycin and glutamine. MTT solution 20 µl was added to 100 µl cell suspension in 96 well plates and incubated for 24 h at 37 °C. The formazan crystals were dissolved with 0.1 N HCL solution and the absorption was determined by an ELISA reader at 570 nm.

Flow cytometry based detection of apoptosis

Apoptotic PBMC were detected with the Annexin V-FITC/PI Apoptosis detection kit (Pharmingen Cat.No. 6693KK). A 100 µL aliquot cell of suspension was incubated with 5 µl Annexin V-FITC and 2 µl propidium iodide (PI) for 15 min. After addition of 400 µl binding buffer, PBMC were analyzed by dual wavelength flow cytometry within 1 h.

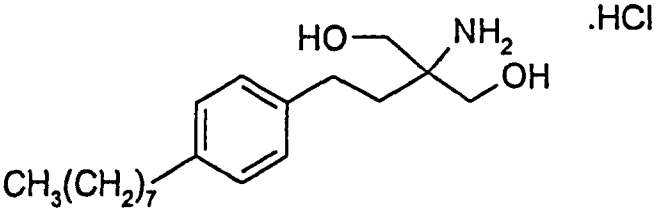

Fig. 1 FTY 720A is a derivative of the sphingosine myriocin (ISP-1) produced by *Isaria sinclairii*

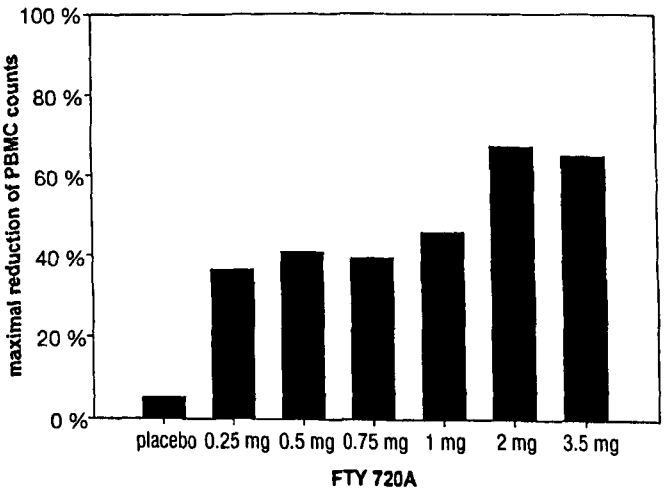

Fig. 2 FTY 720A dose dependently reduced PBMC in stable renal allograft recipients

Results

FTY 720A dose dependently reduced peripheral lymphocyte counts in human renal allograft recipients.

Within 2–6 h after intake of FTY 720 A (0.25–3.5 mg; PO), we observed a significant ($P < 0.01$) and dose-dependent reduction of the number of blood lymphocytes. Lymphycyte counts were reduced by 40–65% compared to 5% in patients who received placebo. FTY 720A doses up to 2 mg resulted in decreased lymphocyte counts which returned to normal after 12–24 h. A dose of 3.5 mg FTY 720 caused lymphopenia which persisted for at least 96 h (Fig. 1). FTY 720A did not affect apoptosis rates in human renal allograft recipients.

We found apoptosis rates of between 2% and 3% before treatment and 2 h, 6 h, 10 h, 24 h and 96 h post-treatment. Apoptosis rates did not change after intake of FTY 720A and were not increased compared to placebo control (Fig. 2).

In vitro, high concentrations of FTY 720A induced apoptosis of human PBMC. In vitro experiments with human PBMC revealed that 10 µM FTY 720A induced 25% apoptosis. In parallel, cell viability decreased to 75% (Fig. 3).

Discussion

In the first human trial we confirmed preclinical data that FTY 720 A causes reversible lymphopenia. We found a significant and dose-dependent reduction of peripheral blood lymphocytes in human renal allograft recipients. Despite its potent immunnosuppressive effects in various animal models, the mechanism of action of FTY 720A is not yet known. Several investigators have reported that FTY 720A reduces lymphocyte counts by apoptosis. We have shown in human renal allograft recipients that FTY 720A (0.25–3.5 mg) did not induce apoptosis in PBMC. This suggests that FTY 720A reduced PBMC by an apoptosis-independent mechanism. However, in vitro, 10 µM FTY720A can induce apoptosis in human lymphocytes from healthy volunteers. In con-

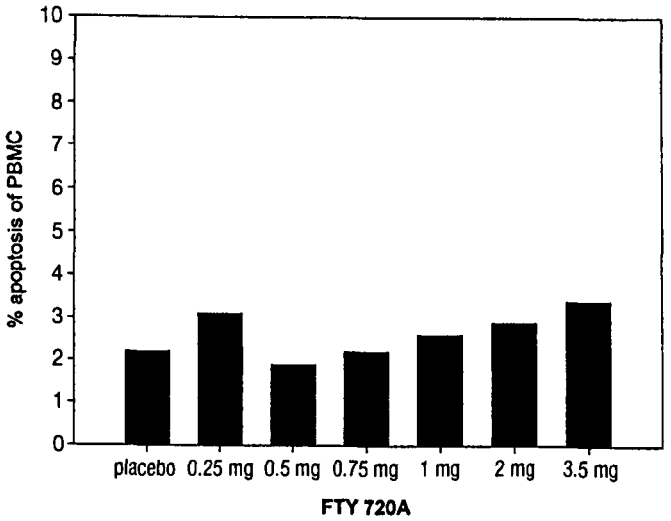

Fig. 3 In stable renal allograft recipients FTY 720A did not affect apoptosis rates of PBMC

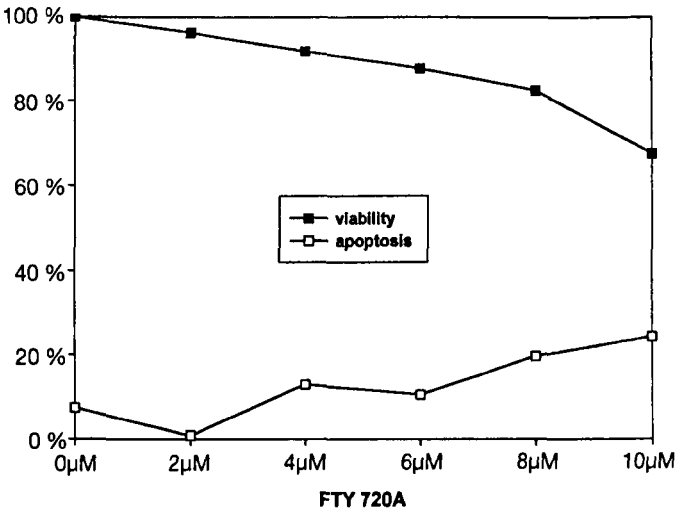

Fig. 4 In vitro, high concentratiions of FTY 720A induced apoptosis of human PBMC

trast, low concentrations of FTY 720A did not induce apoptosis. We suggest that induction of apoptosis in vitro is due to the very high toxic concentration of FTY 720A and has no physiological meaning in the dose range used in our clinical study. In conclusion, our data show that apoptosis does not mediate the caused depletion of peripheral blood lymphocytes caused by FTY 720A and supports the idea that altered lymphocyte homing is the mechanism of action.

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