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Effect of FTY720 on immunoregulation in concordant xenotransplantation

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Abstract The present study was designed to analyze the immunosuppressive activity of FTY720 in concordant xenotransplantation. When T and B lymphocytes of human peripheral blood were incubated with FTY720, the number of viable cells decreased in a dose-dependent manner at doses higher than 4×10^{-5} M. DNA fragmentation was observed at doses higher than 1×10^{-5} M in T cell-rich fractions and at doses higher than 4×10^{-5} M in B cell-rich fractions. These data demonstrate that FTY720 is cytotoxic to B lymphocytes as well as T lymphocytes and apoptosis may play an important role in this cytotoxicity. Golden Syrian hamsters were the donors and Lewis rats the recipients of skin grafts. The recipients were divided into the following four groups: (1) untreated recipients, (2) FTY720 (5 mg/kg per day) was administered orally for 8 days (days −1–6), (3) FK506 (1 mg/kg per day) was injected i.m. for 7 days (days 0–6), and (4) FK506 (1 mg/kg per day) was injected i.m. for 7 days (days 0–6) and FTY720 (5 mg/kg per day) was administered orally for 8 days (days −1–6). The mean graft survival times in groups 1–4 were 9.7 ± 0.52 days ($n = 6$), 12.0 ± 0.71 days ($n = 6$), 13.2 ± 1.6 days ($n = 6$), and 37.7 ± 4.3 days ($n = 6$), respectively. There was a significant difference in the mean survival time between groups one and four. Combined therapy with FTY720 and FK506 is a useful tool for immunoregulation in xenotransplantation.

Key words Xenotransplantation · FTY720 · FK506 · Apoptosis · Hamster-to-rat

Introduction

FTY720 is a synthetic drug produced by modification of ISP-1, a unique immunosuppressive metabolite from Isaria sinclairii. This induces apoptosis specifically in lymphocytes and prevents the occurrence of allograft rejection in various models of animal organ transplantation [8]. Although the precise mechanism for the immunosuppressive action of FTY720 remains unknown, it is obvious that the drug is classified into a different category from the conventional ones. Because FTY720 does not affect interleukin-2 (IL-2) production from mitogen-stimulated rat spleen cells, its mechanisms of action are distinguishable from those of cyclosporine and FK506, which are known to inhibit IL-2 production from helper T cells [3]. The mechanisms of FTY720 also appear to be distinguishable from those of azathioprine and RS61443 because FTY720 does not inhibit DNA biosynthesis.

In this experiment, the single administration of FTY720 (10 mg/kg p.o.) into normal rats induced a marked reduction of peripheral lymphocytes, and the number of total lymphocytes reached a tenth of the normal level by 6 h after its administration. Because of the extent of lymphocyte reduction after FTY720 administration and the proportion of T and B lymphocytes in peripheral blood, FTY720 appears to have influence on both T and B lymphocytes. This speculation encourages

us to investigate the possible immunosuppressive activity of FTY720 in the immunoregulation of xenotransplantation for which the control of both T and B lymphocytes is essential. The present study was designed to analyze the immunosuppressive activity of FTY720 in concordant xenotransplantation.

Materials and methods

Drugs

FTY720 was provided by Yoshitomi Pharmaceutical Industries (Osaka, Japan). For in vitro culture, FTY720 was dissolved in RPMI-1640 medium (Nissui Chemical, Tokyo, Japan) and adjusted to the experimental concentration. For in vivo use, the drug was dissolved in physiological saline. FK506 was provided by Fujisawa Pharmaceutical Industries (Tokyo, Japan) and was dissolved in physiological saline for in vivo use.

In vivo effect of FTY720 on circulating peripheral leukocytes

FTY720 (10 mg/kg) was orally administered to Lewis rats (Charles River Japan, Yokohama, Japan; $n = 4$). Then the number of peripheral leukocytes and hematocrit were counted periodically.

In vitro effect of FTY720 on T and B lymphocytes

Isolation and culture of human peripheral blood mononuclear cells

Heparinized peripheral blood samples were collected from healthy human volunteers, and mononuclear cells were obtained by Separate-L (Muto Pure Chemicals, Osaka, Japan) density-gradient centrifugation. Peripheral blood mononuclear cells were suspended in RPMI-1640 medium containing 10% fetal bovine serum (FBS) (Gibco, Grand Island, N. Y., USA) and nylon wool fractionation was performed. In this way, mononuclear cells were separated into T cell-rich and B cell-rich fractions. These cells, suspended in RPMI-1640 medium containing 5% human AB serum at a concentration of 1×10^6/ml, were incubated for 4 h at 37 °C in a 5% CO_2 atmosphere with or without FTY720 at doses of 1×10^{-7}, 1, 2, 4, 6, 8×10^{-6}, 1, 2, 4, 6, 8×10^{-5}, and 1×10^{-4} M, respectively. Then viable cells, judged by the trypan blue dye exclusion test, were counted.

Agarose gel electrophoresis

Incubated T and B cells were collected and genomic DNA was prepared by the standard SDS-proteinase K digestion/phenol–chloroform extraction method [2]. Genomic DNA was analyzed by electrophoresis in 1.2% agarose gel containing 0.5 μg/ml of ethidium bromide.

Hamster-to-rat skin transplantation

Golden Syrian hamsters weighing 100–150 g (Inoue Laboratories, Kumamoto, Japan) were the donors and Lewis rats ($RT1^l$) weighing 200–300 g were the recipients. Skin grafts were transplanted onto the back of recipients and bandages were applied until day 7 after grafting.

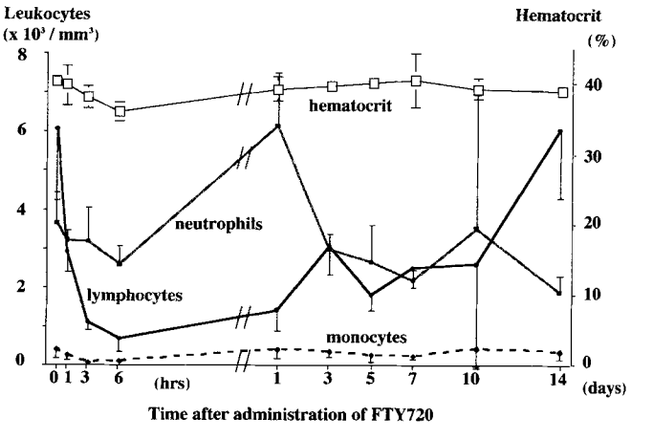

Fig. 1 In vivo effect of FTY720 on circulating peripheral leukocytes

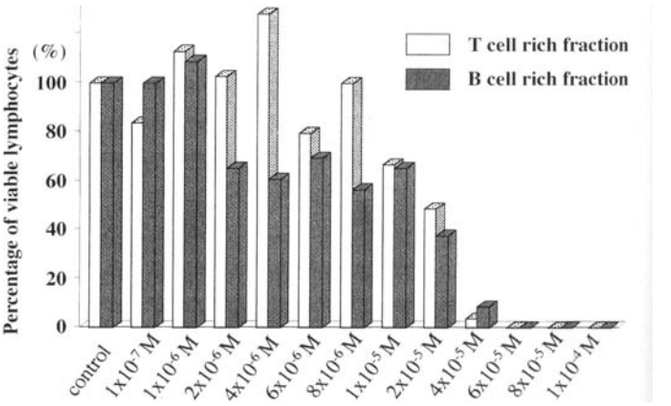

Fig. 2 In vitro effect of FTY720 on T and B leukocytes

Experimental design

The recipients were divided into the following four groups: (1) untreated recipients, (2) FTY720 (5 mg/kg per day) was administered orally for 8 days (days −1–6), (3) FK506 (1 mg/kg per day) was injected i.m. for 7 days (days 0–6), and (4) FK506 (1 mg/kg per day) was injected i.m. for 7 days (days 0–6) and FTY720 (5 mg/kg per day) was administered orally for 8 days (days −1–6). Skin grafting was performed on day 0.

Statistical analysis

The differences in graft survival between experimental groups were analyzed by the generalized Wilcoxon test. A probability value below 0.05 was considered statistically significant.

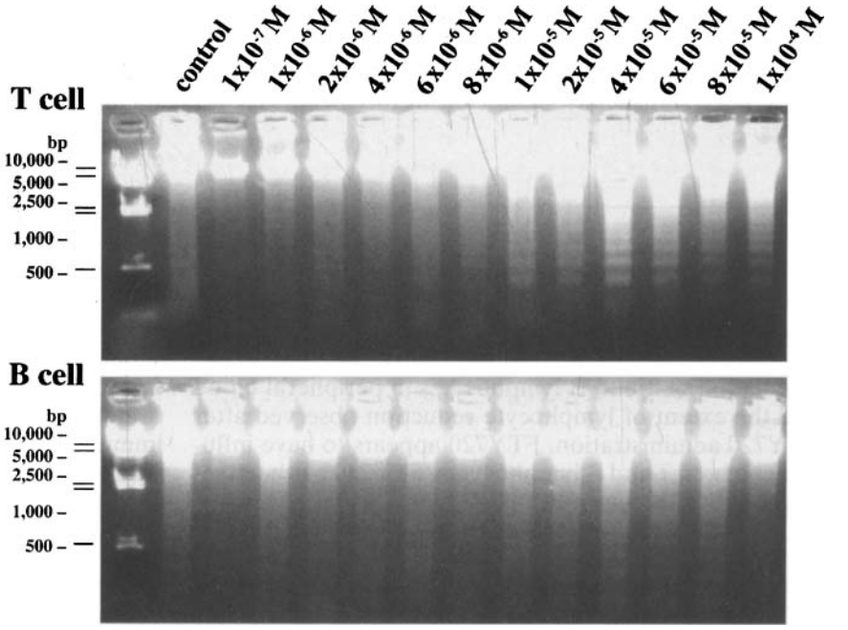

Fig. 3 Selective analysis of T and B leukocytes apoptosis induced by FTY720

Results

In vivo effect of FTY720 on circulating peripheral leukocytes

After the administration of FTY720 at a dose of 10 mg/kg orally to Lewis rats, the number of peripheral lymphocytes began to decrease immediately, and reached less than 1000/mm^3 h after administration (Fig. 1). Then this level increased gradually to a normal level 14 days after administration. However, the number of peripheral neutrophils and monocytes did not decrease, and neutrophils temporarily increased 1 day after administration. The hematocrit level and body weight of the rats remained constant during the observation period.

Selective analysis of T and B cell apoptosis induced by FTY720

When peripheral T and B lymphocytes were incubated for 4 h with FTY720 at concentrations of 1×10^{-7}, 1, 2, 4, 6, 8×10^{-6}, 1, 2, 4, 6, 8×10^{-5}, and 1×10^{-4} M, the number of viable cells decreased in a dose-dependent manner at doses higher than 4×10^{-5} M (Fig. 2). Genomic DNA prepared from these peripheral T and B lymphocytes was analyzed by electrophoresis in 1.2% agarose gel. DNA fragmentation, which is one of the adequate evidences of apoptosis, was observed at doses higher than 1×10^{-5} M in the T cell-rich fraction and higher than 4×10^{-5} M in B cell-rich fraction (Fig. 3).

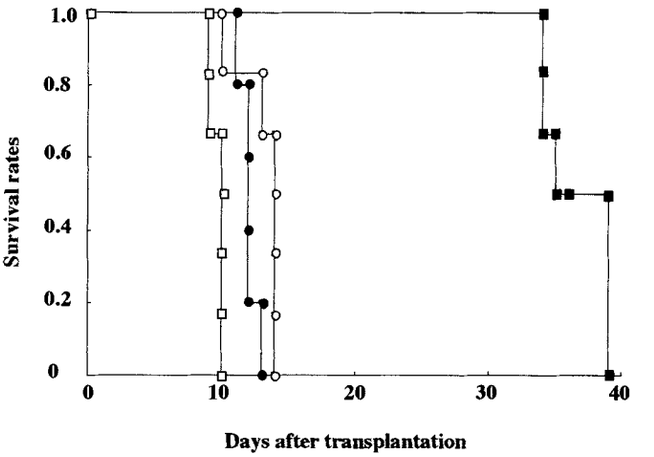

Fig. 4 Graft survival curves after hamster-to-rat skin xenotransplantation. Group 1 (□): untreated recipients ($n = 6$), group 2 (●): recipients treated with single drug therapy of FTY720 ($n = 6$), group 3 (○): recipients treated with single drug therapy of FK506 ($n = 6$), group 4 (■): recipients treated with FTY720 combined with FK506 ($n = 6$)

Hamster skin graft survival times in rat recipients

In the untreated group 1, the mean graft survival time of recipients was 9.7 ± 0.52 days ($n = 6$; Fig. 4). The mean graft survival times of recipients treated with the single drug therapies of FTY720 (group 2) and FK506 (group 3) were 12.0 ± 0.71 days ($n = 6$) and 13.2 ± 1.6 days ($n = 6$), respectively. Neither FTY720 nor FK506 therapy alone induced significant prolongation of graft sur-

vival. However, when the recipients were treated with FTY720 combined with FK506 (group 4), graft survival was markedly prolonged to 37.7 ± 4.3 days ($n = 6$). They were statistically significant in the mean survival times.

Discussion

The in vivo single administration of FTY720 dramatically reduced the peripheral lymphocytes, but not neutrophils or monocytes. It is evident that FTY720 specifically affects lymphocytes in peripheral blood. Because of the population of T and B lymphocytes in peripheral blood and the extent of lymphocyte reduction observed after FTY720 administration, FTY720 appears to have influence on both T and B lymphocytes. FTY720 has been reported to affect specifically T lymphocytes, in which CD4 positive cells are the most sensitive to the drug [3, 4]. This postulation is based on the results obtained from the rat in vivo study showing that the number of CD3-positive T cells in peripheral blood was remarkably decreased, whereas the number of CD45RA-positive B cells was not affected, with treatment of FTY720 at a dose of 0.1 mg/kg for 14 days [3, 4]. In contrast with this previous study, we used the relatively high dose of FTY720 (10 mg/kg) in in vivo studies evaluating the effect of FTY720 on circulating peripheral leukocytes. It is possible that the susceptibility of T and B lymphocytes to FTY720 depends on the administered dosage.

To address the susceptibility of T and B lymphocytes to FTY720, FTY720-induced T and B lymphocyte death was separately analyzed in the present study. In both peripheral T and B lymphocytes incubated with FTY720, the number of viable cells decreased in a dose-dependent manner, and DNA fragmentation was observed at doses higher than 1×10^{-5} M in the T cell-rich fractions and at doses higher than 4×10^{-5} M in the B cell-rich fractions. These data demonstrate that FTY720 is cytotoxic to B lymphocytes as well as T lymphocytes and apoptosis may play an important role in this cytotoxicity. It has been suggested that FTY720 displays *bcl-2*-associated apoptotic cell death in human mononuclear cells [9]. Taking into account that clonal deletion of mature self-reactive B lymphocytes is related to *bcl-2*-associated apoptosis [7], it is likely that the peripheral B lymphocytes are susceptible to the apoptosis-inducing drug.

The cytotoxicity of FTY720 to both T and B lymphocytes encouraged us to investigate the possible immunosuppressive activity of this drug in regulating the xenotransplantation immune response for which the control of both T and B lymphocytes is essential. To assess the immunosuppressive activity of FTY720 in xenotransplantation, we used the hamster-to-rat model which is designated as a concordant combination. In the hamster-to-rat model, FK506 is likely to be the most effective immunosuppressive drug currently available, and it is known that the combined therapy with FK506 and various immunosuppressive drugs can improve xenograft survival [1, 5, 6]. In the present study, skin xenograft survival was not improved when FTY720 and FK506 were employed separately; however, a combination of the two drugs improved the survival significantly ($P < 0.05$). FTY720 possesses distinct mechanisms from FK506, i.e., FTY720 does not affect IL-2 production from antigen-stimulated T lymphocytes, whereas FK506 inhibits IL-2 production from activated T lymphocytes [3]. Also, FTY720, unlike FK506, is cytotoxic to B lymphocytes. Taken together, we postulate that FTY720 combined with FK506 shows a synergistic effect on skin xenograft survival. Considering the lesser toxicity of FTY720, this regimen may be beneficial when xenotransplantation reaches the stage of clinical applicability.

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