

Effects of FK506 on the Growth of Freshly Isolated Parenchymal Hepatocytes and Renal Cortical Tubular Cells

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FK506 is an immunosuppressive agent used in kidney and liver transplantation. After transplantation, kidney or liver usually starts hyperplasia or regeneration supported by cellular growth. The effects of FK506 on the growth of freshly isolated rat parenchymal hepatocytes or rabbit renal cortical tubular cells in culture were examined within a concentration range of 10 μ M. FK506 inhibited the DNA synthesis of cultured hepatocytes and renal tubular cells in a dose-dependent manner. This was not due to increase of release of the activated transforming growth factor- β . The cytotoxic effect of FK506 on the cells was examined, but no significant increase in the release of N-acetyl- β , D-glucosaminidase (NAG) from tubular cells or of glutamic oxaloacetic transaminase (GOT) from hepatocytes was observed. FK506 at a non-cytotoxic concentration thus appears to directly inhibit the growth of cultured hepatocytes and tubular cells *in vitro*.

INTRODUCTION

FK506 is a potent immunosuppressive agent used for human organ transplantation to protect graft rejection reaction. FK506 has been isolated from a streptomycetes¹⁾. At the time of removal of a donor kidney, transient ischemia occurs, causing acute tubular necrosis (ATN). The hepatocyte growth factor (HGF) may possibly be essential to the regeneration of tubular cells from ATN²⁾. A graft causes compensatory renal growth accompanied by transient tubular cell hyperplasia³⁾. Transplanted liver also causes parenchymal hepatocyte regeneration. Following organ transplantation, patients must receive immunosuppressive therapy to avoid graft rejection. The effect of immunosuppressive agent on hyperplasia and regeneration of cells⁴⁻⁶⁾ are little understood. The direct effects of FK506 on the growth of parenchymal hepatocytes and renal cortical tubular cells in a simple cell culture system were examined in this study.

MATERIALS AND METHODS

Preparation of FK506 solution.

FK506 was kindly provided by Fujisawa Pharmaceuticals, Osaka, Japan. In 5 mM stock solution, FK506 was dissolved in ethanol and it was diluted with culture medium when it was used for the assays.

Preparation of HgCl₂ solution.

HgCl₂ (Wako Pure chemicals, Osaka, Japan) was dissolved in saline and 10⁻³ M stock solution was prepared. At the time of use, stock solution was diluted with culture medium.

Isolation and culture of adult rat parenchymal hepatocytes.

The method has already been reported⁷⁾. Parenchymal hepatocytes were isolated by *in situ* collagenase perfusion. Isolated hepatocytes contained few non-parenchymal cells and were cultured in serum-free Williams' E medium supplemented with 10⁻⁹ M insulin and 10⁻⁹ M dexamethasone, and 5 U/ml aprotinin. Cell density in DNA synthesis was 6.25 \times 10⁴ cells/cm² and that for collecting the conditioned medium 1.25 \times 10⁵ cells/cm², respectively.

Isolation and culture of rabbit renal cortical tubular cells.

This was done as described previously⁸⁾. Briefly, a homogenate of rabbit renal cortices was filtered through 245 μ m-pore and 105 μ m-pore nylon meshes. Remnant tubular fragments on the latter mesh, which contained few glomeruli, were cultured in collagen-coated culture dishes in basal medium (a 50:50 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium containing 20 ng/ml piperacillin and 5 U/ml aprotinin) supplemented with 10⁻⁸ M insulin, 10⁻⁸ M dexamethasone, and 5 μ g/ml bovine transferrin. The tubular cells contained few glomerular cells or fibroblasts.

Assay of the DNA synthesis of rat hepatocytes and rabbit renal cortical tubular cells.

Assays of the DNA synthesis of cultured rat parenchymal hepatocytes and rabbit renal cortical tubular cells were conducted as described previously^{7,8)}. After preparation of the cells, the samples were added with serum- and hormone-free medium to the cells and 16 h (for tubular cells) or 22 h (for hepatocytes), later, [¹²⁵I]-deoxyuridine (final concentration; 37 kBq/ml) was added to the cells and the culture was continued for 4 h at 37 °C. Then, the incorporation of [¹²⁵I]-deoxyuridine into DNA was measured by a gamma counter.

Assay of NAG activity.

NAG is a lysosomal enzyme present in renal tubular epithelial cells. It is released into conditioned medium when cultured tubular cells are damaged. NAG activity in the conditioned medium obtained from primary cultured tubular cells incubated with or without 10 μM of FK506 for 24 h was measured using the assay kit, NAG test Shionogi (Shionogi Pharmaceuticals, Osaka, Japan).

Assay of GOT activity.

GOT is present in parenchymal hepatocytes and released into conditioned medium when cultured hepatocytes are damaged. GOT activity in the conditioned medium from primary cultured parenchymal hepatocytes incubated with or without 10 μM of FK506 for 24 h was measured using the assay kit, GOT-UV test Wako (Wako Pure Chemicals, Osaka, Japan).

Assay of TGF-β activity in the medium conditioned by primary cultured rabbit renal cortical tubular cells and rat parenchymal hepatocytes.

TGF-β activity was determined based on inhibition of the DNA synthesis of cultured rat hepatocytes and was confirmed by the neutralization of the activity using specific antibody against TGF-β 1 and -β 2 (R & D systems, Minneapolis, MN) as described elsewhere⁹⁾. When tubular cells grew subconfluently, the medium was changed to fresh basal medium and incubated with or without FK506 in a CO₂ incubator at 37 °C for 12 h. The cells were then washed 5 times with fresh basal medium to remove FK506 and cultured in basal medium containing 0.1 % bovine serum albumin. Twenty-four hours later, the medium was collected and concentrated 50-fold with a Centricon 10 (Amicon, Danvers, MA). Aliquots of the concentrated conditioned medium were acidified with 0.5 M HCl at room temperature for 2 h, and neutralized with NaOH. TGF-β activity in the concentrated conditioned medium with or without acidification was assayed based

on inhibition of the DNA synthesis of rat hepatocytes stimulated with 10 ng/ml of human recombinant epidermal growth factor (EGF; Wakunaga Pharmaceuticals, Hiroshima, Japan) and 10⁻⁷ M bovine insulin (Sigma Chemical Company, St. Louis, MO). To confirm this activity as TGF-β, aliquots of concentrated conditioned media or native human TGF-β 1 obtained from King Brewery, Kakogawa, Japan were incubated with anti-TGF-β antibody at 37 °C for 1 h and added to cultured hepatocytes. DNA synthesis was then measured. To collect the medium conditioned by rat hepatocytes, freshly isolated hepatocytes were inoculated into a 100 mm dish coated by type I collagen and cultured for 24 h at 37 °C. After being washed, the cells were incubated with Williams' E medium supplemented with 5 U/ml of aprotinin and 0.1 % (W/VO) bovine serum albumin with or without 10 μM FK506. After 12 h-incubation, the cells were washed and cultured in Williams' E medium containing 5 U/ml aprotinin and 0.1 % bovine serum albumin. Twenty-four hours later, the medium was collected and treated as in the case of tubular cells.

RESULTS

Fig. 1 shows the effect of FK506 on the DNA synthesis of primary cultured rat parenchymal hepatocytes. FK506 inhibited the DNA synthesis of hepatocytes dose-dependently with or without insulin and EGF. At 10 μM, FK506 caused no morphological changes in cultured hepatocytes and the suppression of DNA synthesis of hepatocytes was not cytotoxic since there was no increase

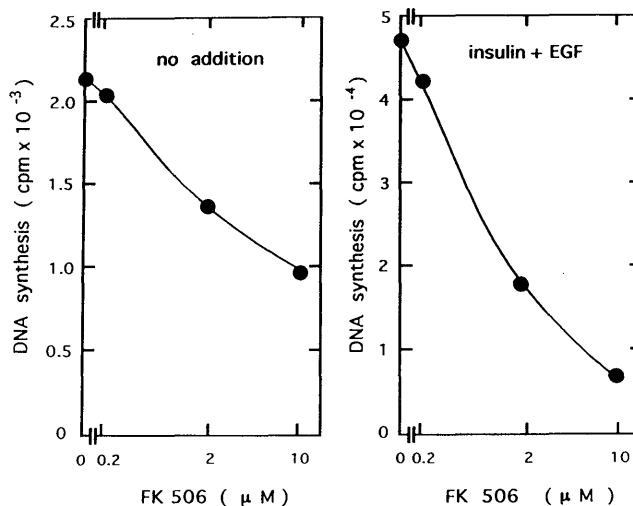


Fig. 1. Effects of FK506 on the DNA synthesis of adult rat hepatocytes in primary culture. FK506 with or without 10⁻⁷M of insulin and 10 ng/ml EGF were added to the cells and the incorporation of iododeoxyuridine was measured as described in "Materials and Methods". Values are means for triplicate experiments.

Table 1. Release of enzyme from cultured parenchymal hepatocytes and renal cortical tubular cells.

(I) Hepatocytes (n=3)

FK506(μ M)	GOT(IU/ 1×10^5 cells)
0	2.57 ± 0.33
1	3.03 ± 0.31
10	2.77 ± 0.98

(II) Tubular cells (n=5)

FK506(μ M)	NAG(U/ 1×10^6 cells)
0	2.83 ± 2.15
10	1.16 ± 0.98

Experimental conditions are as described in "Materials and Methods". Values are expressed as means \pm SD.

Table 2. Effects of anti TGF- β antibody on medium conditioned by cultured rat hepatocytes.

Addition	DNA synthesis of hepatocytes (cpm)
none	2432 ± 156
I + E	20831 ± 1234
I + E + control IgG	22317 ± 1956
I + E + anti TGF- β	21543 ± 2021
I + E + hTGF- β	2020 ± 235
I + E + hTGF- β + control IgG	2220 ± 216
I + E + hTGF- β + anti TGF- β	18572 ± 895
I + E + acid-CM	3032 ± 455
I + E + acid-CM + control IgG	2906 ± 180
I + E + acid-CM + anti TGF- β	19183 ± 1033

Experimental conditions are as described in "Materials and Methods". I, insulin 10^{-7} M; E, EGF 10 ng/ml; control IgG, non-immune rabbit IgG 50 mg/ml; anti TGF- β , anti TGF- β rabbit IgG 50 μ g/ml; hTGF- β , human TGF- β 11 ng/ml; acid-CM, 20% (Vo/Vo) of acidified and then neutralized conditioned medium. Values are means \pm SD for triplicate experiments.

in the release of GOT in the medium (Table 1). TGF- β is a potent growth inhibitor of cultured hepatocytes¹⁰. The effect of FK506 on the secretion of TGF- β activity by cultured hepatocytes was investigated. Conditioned medium was collected after washing and the removal of FK506 because of its inhibitory effect on the DNA synthesis of hepatocytes. Fig. 2 shows the acidified medium conditioned by rat hepatocytes to inhibit the DNA synthesis of hepatocytes dose-dependently. To confirm this inhibitory effect of acidified conditioned medium, the effect of anti-TGF- β antibody on the activity was investigated. The inhibitory activity was noted to be neutralized by the specific antibody against TGF- β (Table 2). This indicates that the inhibition of DNA synthesis of hepatocytes is due to TGF- β activity and not to

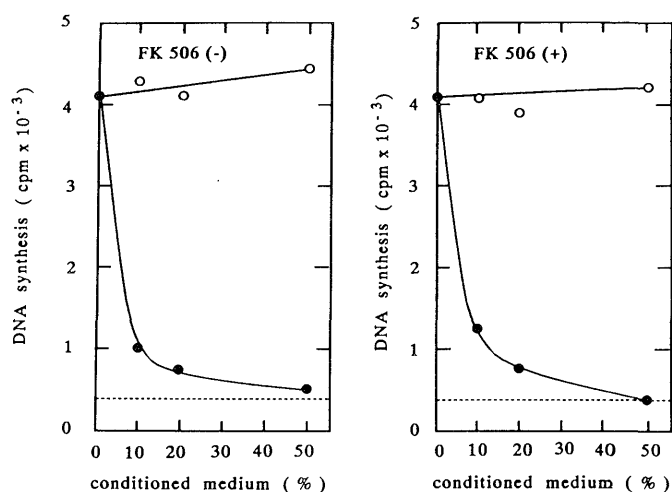


Fig. 2. Effects of the medium conditioned by rat hepatocytes treated with or without FK506 on the DNA synthesis of cultured rat hepatocytes stimulated by 10^{-7} M insulin and 10 ng/ml EGF. Experimental conditions are as described in "Materials and Methods". Open circle, non-treated conditioned medium; closed circle, acidified conditioned medium; dotted line, basal DNA synthesis. Values are expressed as means for triplicate experiments.

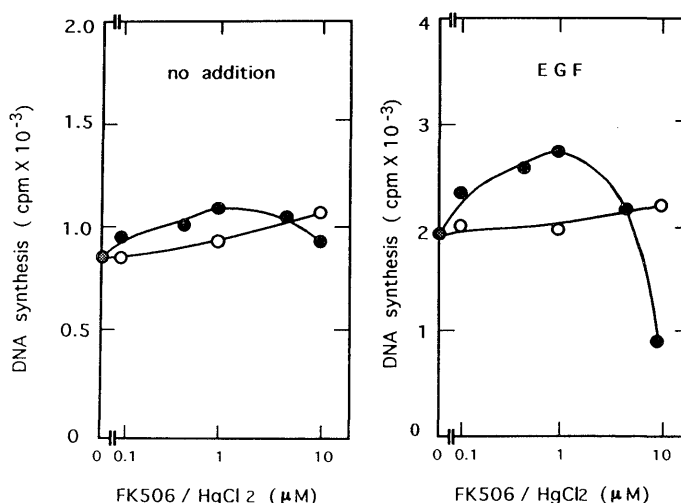


Fig. 3. Effects of FK506 and HgCl₂ on the DNA synthesis of cultured rabbit renal cortical tubular cells. FK506 and HgCl₂ with or without 10 ng/ml EGF were added to the cells and the incorporation of iododeoxyuridine was measured as described in "Materials and Methods". Open circle, HgCl₂; closed circle, FK506. Values are means for triplicate experiments.

contamination by FK506. Parenchymal hepatocytes secrete TGF- β activity in a latent form and the amount of the activity was not changed with or without FK506 treatment.

The effects of FK506 and HgCl₂, which is known to cause ATN²), on the DNA synthesis of cultured rabbit renal cortical tubular cells were studied. Fig. 3 shows that FK506 inhibits the DNA synthesis of tubular cells only at

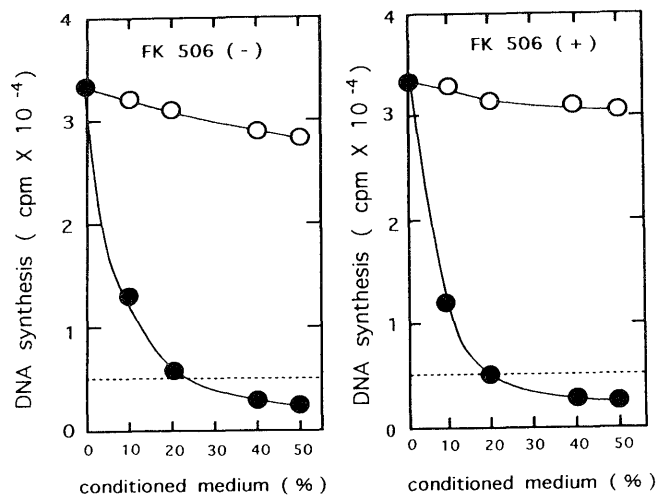


Fig. 4. Effects of the medium conditioned by renal tubular cells treated with or without FK506 on the DNA synthesis of cultured rat hepatocytes stimulated by 10^{-7} M insulin and 10 ng/ml EGF. Experimental conditions are as described in "Materials and Methods". Open circle, non-treated conditioned medium; closed circle, acidified conditioned medium; dotted line, basal DNA synthesis. Values are expressed as means for triplicate experiments.

a high concentration (10 μ M). But no remarkable effect of HgCl₂ was observed at the same concentration and the inhibitory potency of FK506 appeared more strong than that of HgCl₂. FK506 affects no morphological change in cultured tubular cells at 10 μ M and had no cytotoxic effect at the same concentration (Table 1). The effect of FK506 on the secretion of TGF- β activity by cultured tubular cells was investigated. To assess TGF- β activity, we used cultured hepatocytes whose growth is inhibited by FK506. The medium conditioned by tubular cells used following incubation with FK506, and the removal of FK506 was used. Fig. 4 shows the inhibitory effect of the

Table 3. Effects of endothelin-1 (ET-1) on the DNA synthesis of cultured rabbit renal cortical tubular cells.

Addition	DNA synthesis(cpm)
none	973 \pm 2
I + E	4397 \pm 100
ET-1 10^{-10} M	1005 \pm 14
ET-1 10^{-9} M	1021 \pm 35
ET-1 10^{-8} M	994 \pm 23
ET-1 10^{-7} M	1038 \pm 51
I + E + ET-1 10^{-10} M	4332 \pm 26
I + E + ET-1 10^{-9} M	4118 \pm 45
I + E + ET-1 10^{-8} M	3990 \pm 88
I + E + ET-1 10^{-7} M	4250 \pm 74

Human endothelin-1 (Peptide Institute Inc., Minoh, Japan) was added to cultured rabbit renal cortical tubular cells and the DNA synthesis was measured as described in "Materials and Methods". Values are expressed as means \pm SD for triplicate experiments.

conditioned medium on the DNA synthesis of cultured tubular cells in the case of acidification. This activity was also neutralized by anti-TGF- β antibody (data not shown). Secreted TGF- β activity appeared latent, as reported previously¹¹). No obvious alteration in the amount of secreted both latent and active form TGF- β activity could be detected. It has been reported that endothelin-1 (ET-1) is secreted by cultured tubular cells following stimulation with FK506⁹. To determine whether ET-1 is an autocrine growth stimulating factor, the effect of ET-1 on the DNA synthesis of cultured tubular cells was investigated. ET-1 had no effect on the DNA synthesis of cultured tubular cells with or without insulin and EGF (Table 3).

DISCUSSION

FK506 at high concentration inhibited the DNA synthesis of freshly isolated tubular cells and parenchymal hepatocytes in culture, but the effect was not cytotoxic. The inhibitory effect of FK506 on the growth of hepatocytes was more potent than that on tubular cell growth, and that on the tubular cell growth, more potent than that of HgCl₂. The concentration of blood FK506 at the concentration in clinical use is usually less than 0.1 μ M¹²). The regeneration and hyperplasia of renal tubular cells or parenchymal hepatocytes apparently is not inhibited by FK506 at this concentration.

Rao et al.¹³) reported mRNA of TGF- β to increase after FK506 administration in vivo, but no increase of TGF- β activity secreted in the medium conditioned by parenchymal hepatocytes or tubular cells was observed in this study. Total cellular mRNA used in their study contained mRNA from non-parenchymal liver cells and increase in TGF- β mRNA may have been caused by non-parenchymal cells. Sakr et al.¹⁴) found FK506 to prevent renal ischemia/reperfusion injury. This may have been due to inhibition of the production of tumor necrosis factor by FK506. But no growth stimulatory or inhibitory effect of the tumor necrosis factor on the growth of cultured renal tubular cells was observed¹⁵). Moutabarrick et al.⁶) reported that cultured tubular cells produce ET-1 in response to FK506. But ET-1 has no effect on the growth of tubular cells. It is thus apparent that growth inhibition of FK506 is not mediated by known cytokines such as TGF- β , tumor necrosis factor, or ET-1.

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