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Tacrolimus causes a blockage of protein secretion which reinforces its immunosuppressive activity and also explains some of its toxic side-effects

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ABSTRACT

Background: Tacrolimus (FK506) is a macrolide immunosuppressant drug from the calcineurin inhibitor family, widely used in solid organ and islet cell transplantation, but produces significant side-effects.
Objective: This study examined the effect of FK506 on interleukin-2 (IL-2) and insulin secretion, establishing a novel characteristic of this drug that could explain its diverse adverse effects, and developed an experimental model for the simultaneous analysis of mRNA expression and protein secretion affected by this drug.
Methods: The IL-2 levels when tacrolimus was administered were analysed by Western blot, immunocytochemistry and RT-PCR in a T lymphocyte cellular line (Jurkat) 24 h post-stimulation. The insulin levels when tacrolimus was administered were analysed 4 h after stimulation of glucose-induced insulin secretion in a pancreatic cellular line (MIN6).
Results: The previously published information describes tacrolimus as only capable of partially blocking IL-2 mRNA expression. In our hands, the cellular content of IL-2 is almost undetectable in stimulated Jurkat cells and can be detected in cellular extracts only when the secretory pathway is blocked by brefeldin A (BFA). BFA added 2 h after the beginning of stimulation was able to inhibit IL-2 secretion, without affecting IL-2 mRNA expression. Therefore BFA utilization allowed us to establish a model to analyze the effect on IL-2 secretion, separately from its expression. Tacrolimus added before stimulation inhibits only IL-2 synthesis, but blocks IL-2 protein secretion when added 2 h after stimulation. Similarly, tacrolimus is also capable of blocking the glucose-stimulated secretion of insulin by MIN6 cells. An increase of the intracellular content can be detected concomitantly with a decrease of the hormone measured in the culture medium.
Conclusions: Results of this study indicate that tacrolimus possesses another important effect in addition to the inhibition of IL-2 gene transcription, namely the ability to act as a general inhibitor of the protein secretory pathway. These results strongly suggest that the diabetogenic effect of the immune suppressant FK506 could be caused by the blockade of insulin secretion. This novel effect also provides an explanation for other side-effects observed in immunosuppressive treatment.

1. Introduction

The immunosuppressive drugs tacrolimus (FK506) and cyclosporine (CsA) belong to the class of immunosuppressant agents referred as calcineurin inhibitors, based on their proposed action mechanism and have been widely used in organ and cell transplantation and more recently in autoimmune diseases [1,2]. Several studies have demonstrated that tacrolimus exerts its immunosuppressive effects primarily by interfering with the activation of T cells [3,4]. The macrolide FK506 is a potent immunosuppressant that canonically inhibits a key step in T cell activation, blocking the interleukin-2 (IL-2) gene transcription [5,6].

This process is initiated by the binding of tacrolimus to the cytoplasmic immunophilins FKPBs, where the isoform FKBP12 is the main effector in the immunosuppressive effect caused by tacrolimus [6–8]. The tacrolimus–FKBP12 complex inhibits the activity of calcineurin, a serine–threonine phosphatase that regulates the IL-2 promoter induction after T cell activation [9,10]. This inhibition of calcineurin impedes the calcium-dependent signal transduction, and inactivates the transcription factors (NF-AT's) that promote cytokine gene activation, as these are direct or indirect substrates of the calcineurin's serine–threonine phosphatase activity [11,12]. As a consequence, the transcription of cytokines IL-2, IL-3, IL-4, IL-5, interferon-γ, tumor necrosis factor-α, granulocyte-macrophage colony-stimulating factor and IL-2 and IL-7 receptors is suppressed by tacrolimus [3,13–16].

Another calcineurin inhibitor, cyclosporine A (CsA), exerts similar inhibitory effects on inflammatory cytokines, although the inhibitory effect of CsA is less potent than that of tacrolimus [17]. This widely accepted mechanism, however, does not readily explain the different side-effects caused by this drug (diabetogenesis, neuropathy and nephrotoxicity). [18–23].

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Post-transplant diabetes mellitus (PTDM) is a known, common side-effect of treatment with several immunosuppressive drugs [24] and often leads to the need for exogenous insulin administration to normalize glucose homeostasis in graft recipients [25]. Obviously, the diabetogenicity of immunosuppressive drugs is particularly undesiderable for pancreas and islet transplantation. Indeed, the limited survival of initially successful islets allograft, particularly in the past, may be partially explained by the diabetogenicity of the immunosuppressive regimens utilized [26]. The toxic effects observed in several tissues, such as pancreatic and central nervous system cells, remain however unexplained.

Several studies have investigated the effects of these immunosuppressive agents on beta-cell function in human cell lines [27], rodent beta cells [28] and human beta cells [29–31]. These studies have shown that high doses of FK506 can cause significant exocytosis of cellular insulin [27], inhibition of insulin secretion upon stimulation by glucose [28,31], induction of apoptosis in both beta and alpha cells [29] and abolished beta-cell regeneration [32]. *In vivo* treatment of different animal species with oral doses of tacrolimus of at least 1 mg/kg/day, induced glucose intolerance after 2 weeks but no hyperglycemia [33–39]. The glucose-intolerant animals showed a decreased pancreatic insulin content [34], vacuolation and degranulation of the isolated islets [33], an increased rate of islet apoptosis [39] or a decreased immunostaining of insulin, together with a diminished *in situ* hybridization of insulin mRNA in beta cells [38].

It is therefore clear that the FK506 immunosuppressive treatment must affect other equally important cellular pathways, different from the canonical action mechanism dependent on the transcriptional inhibition of IL-2. As an example, despite the fact that FK506 is a powerful immunosuppressive drug that inhibits the activation of several transcription factors (nuclear factors NF-AT and NF-kB) critical for T cell activation, FK506 administration induces the NF-kB-regulated IL-6 transcription *in vitro* and *in vivo* in kidney, which could probably explain the nephropathy often observed during the immunosuppressive treatment [40].

Previous studies have correlated the immunosuppressive effect of these drugs with the inhibition of IL-2 expression, measuring calcineurin activity and IL-2 transcription levels. We have extended these determinations to include the effects of these drugs on protein secretion in different cell lines. We show that in the T lymphocyte cell line Jurkat J77, FK506 alters the secretion of this cytokine, similarly to the effect of brefeldin A (BFA), in addition of the expected transcriptional inhibition. Additionally, we have demonstrated that FK506 treatment also blocks the insulin secretion in the pancreatic cell line MIN6 as early as 4 h post-glucose stimulation.

We therefore propose a novel effect for the immunosuppressive drug FK506, which clearly blocks IL-2 protein secretion in Jurkat cells. By extension, the diabetogenic side-effect can be explained by the blockage of insulin secretion in MIN6 pancreatic cells.

2. Methodology

2.1. Chemical compounds

Tacrolimus (10 mg/ml; Tecoland Labs), CsA (1 mg/ml; MP Biomedicals LLC) and BFA (100 μM; Sigma-Aldrich) were dissolved in DMSO (Aldrich Chemicals, Milwaukee, WI, USA) and stored at 4 °C. An equivalent DMSO volume was used as a control when indicated.

2.2. Cell lines and tissue culture

Jurkat J77 cells were grown in RPMI 1640, supplemented with 10% bovine calf serum, 100 U of penicillin/streptomycin and 5 mM glutamine at 37 °C in 5% CO_2/O_2.

Mouse insulinoma (MIN6) cells were used between passages 16 and 35 at 80% confluence. MIN6 cells were grown in DMEM containing 25 mM glucose (DMEM full media) supplemented with 15% heat-inactivated fetal calf serum, 100 μg/ml streptomycin, 100 U/ml penicillin sulfate, and 75 μM β-mercaptoethanol, equilibrated with 5% CO_2, 95% air at 37 °C. Prior to treatment, the medium was removed and the cells washed twice with HEPES-balanced Krebs–Ringer bicarbonate buffer (115 mM NaCl, 5 mM KCl, 10 mM $NaHCO_3$, 2.5 mM $MgCl_2$, 2.5 mM $CaCl_2$, 20 mM HEPES, pH 7.4) containing 0.5% bovine serum albumin (KRB buffer).

2.3. IL-2 and insulin secretion

Secretion of IL-2 was induced by incubating 10^6 Jurkat cells with 10 ng/ml of phorbol 12-myristate 13-acetate (PMA) and 2% of phytohemagglutinin (PHA) in a final volume of 800 μl as described previously [41]. The total duration of stimulation was varied in different experiments (0 to 48 h) and is specified in the Results section and the appropriate figures.

To determine the secreted and intracellular IL-2, cells were harvested by centrifugation (250 ×*g* for 10 min), the cell-free medium supernatant was collected and the Jurkat cells were washed twice with PBS to remove the secreted IL-2. Then the cells were lysed using 1.0% Triton X-100 and suspended in 100 μl of PBS. The supernatant and lysed cells were analyzed by Western blot.

Secretion of insulin was measured by plating 10^5 MIN6 cells per well of a 6-well plate in 1 ml of complete media and grown for 3 or 4 days. Media were replaced with KRB buffer and the cells were then incubated for 2 h at 37 °C in KRB buffer prior to incubation in KRB buffer or DMEM containing 5 or 25 mM glucose for a further hour with or without drugs at various concentrations at 37 °C for 15 min, followed by buffer alone at the times indicated in each figure. Cell viability was monitored at timed intervals using Trypan blue exclusion as previously described [42]. In all cases the viability of the cells was 98–100%. The insulin released into the KRB or DMEM was then analysed by ELISA and corrected with an average cell count determined for each well as described above.

2.4. Immunoblotting

Protein quantitation was performed by Western blot analysis. The samples were separated using 10% discontinuous SDS-PAGE. The resolved membrane proteins were transferred to Immobilon membranes (Millipore, Bedford, MA, USA) and then soaked in 5% non-fat dried milk in Tris–buffered saline containing Tween-20 (TBS-T; 10 mM Tris–HCl, pH 7.2, 250 mM NaCl, 0.05% Tween-20) at 4 °C overnight. The membrane was incubated with rabbit anti-IL-2 polyclonal primary antibody (1:1000 dilution in PBS-T) at room temperature for 1 h, then incubated with a biotinylated secondary antibody (1:5000 dilution in PBS-T) at room temperature for 1 h, and developed with peroxidase-conjugated streptavidin at room temperature for 1 h. Specific bands were visualized by ECL (enhanced chemiluminescence; Amersham Biosciences, Arlington Heights, IL) [43]. As controls, membranes were incubated with antibodies pre-absorbed with the respective peptide used to generate the antibodies.

2.5. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNAs were isolated by the method of Chomczynski and Sacchi [44] from Jurkat J77 cells. Only RNA samples that yielded intact 18S and 28S bands with the expected band ratio were included in subsequent experiments. The reverse transcription reaction was performed in a reaction mixture of 20 μl total volume containing 2 μg total RNA of each sample, 200 U Moloney murine leukaemia virus reverse transcriptase (BioLabs, New England), 1 mM each of the dNTPs (dATP, dCTP, dTTP, and dGTP), 20 U of ribonuclease inhibitor, 0.5 mg oligo (dT) primer, 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 10 mM dithiothreitol, and 3 mM $MgCl_2$. The RT mix was incubated in a