Stimulation of transforming growth factor-β_1 transcription by cyclosporine

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Abstract In searching for a candidate mechanism for the immunosuppressive as well as fibrogenic consequences of cyclosporine usage, we have explored the hypothesis that cyclosporine stimulates transcription of transforming growth factor-β_1 (TGF-β_1), a multifunctional cytokine endowed with immunosuppressive and fibrogenic properties. Our results demonstrate that cyclosporine (i) stimulates TGF-β_1 promoter-dependent transcription of chloramphenicol acetyl transferase gene in transiently transfected human A-549 cells, (ii) stimulates the synthesis of TGF-β_1 RNA transcripts in human T cells, and (iii) permits the expression/emergence of DNA regulatory proteins (retinoblastoma control factor-1 (RCF-1) and RCF-2) that bind and regulate TGF-β_1 promoter activity. Our studies demonstrate for the first time that cyclosporine stimulates TGF-β_1 gene transcription and suggest a novel mechanism of action of cyclosporine.

Key words: Cyclosporine; TGF-β; Transcription; CAT activity; Mobility shift assay; Transcription factor

1. Introduction

Cyclosporine (CsA) is a potent immunosuppressant that has had a significant impact on organ transplantation [1]. A major troubling feature of its clinical usage is fibrosis [1]. In searching for a candidate mechanism for the immunosuppressive as well as fibrogenic consequences of CsA usage, the hypothesis that CsA stimulates transforming growth factor-β_1 (TGF-β_1) gene transcription was explored since TGF-β_1 is not only an immunoregulatory molecule [2,3] but also a fibrogenic cytokine [4]. The scientific underpinning for this provocative postulate (CsA is generally considered to be an inhibitor of gene transcription [5,6]) was provided by our earlier demonstration that CsA increases the steady-state levels of TGF-β_1 messenger RNA (mRNA) as well as TGF-β_1 protein in mammalian cells [7,8].

The TGF-β_1 gene has two well-characterized promoters [9–12]. Elegant studies have shown that at least two DNA binding proteins (retinoblastoma control factors (RCF)-1 and RCF-2) bind to sequences designated as retinoblastoma control elements (RCE) [12]. RCF-1 appears to be stimulator protein-1 (Sp-1) by multiple criteria [12]. It has also been shown that Sp-1 stimulates TGF-β_1 promoter activity [13] and that Sp-1-mediated transcription is regulated by the retinoblastoma gene protein [14].

We report here that (i) CsA stimulates TGF-β_1 transcription and (ii) CsA does not interfere with either the expression of RCF-1 or the emergence of RCF-2 in stimulated normal human T cells. Stimulation of TGF-β_1 gene transcription by CsA represents the first example of an immunoregulatory gene the transcription of which is stimulated in normal human T cells by CsA.

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2. Materials and methods

2.1. Isolation and stimulation of T cells

Highly purified T cells (>98% CD2 antigen-positive cells) were isolated from normal human peripheral blood mononuclear cells and stimulated with a synergistic combination of *sn*-1,2-dioctanoylglycerol (DAG) and ionomycin, as described [7,15]. The T cells were either untreated or pretreated with 100 ng/ml CsA at 37°C for 30 min and then incubated for an additional 1 h at 37°C with 10 μg DAG and 1 μM ionomycin.

2.2. DNA probes

The full-length human IL-2 cDNA plasmid was a gracious gift from Dr. A. Granelli-Piperno, Rockefeller University, New York, NY. Full-length human TGF-β_1 cDNA plasmid phTGFβ-2 was obtained from ATCC (Rockville, MD), plasmid phTG5-CAT was a kind gift of Dr. S.-J. Kim and Dr. Anita Roberts (NCI, NIH), and the plasmid pAct108, carrying exons 1–3 of the rat β actin gene, was a kind gift from Dr. B. Raghu (Washington University, St. Louis, MO).

2.3. TGF-β_1 promoter-dependent transcription

A-549 human adenocarcinoma cells (A-549 cells), obtained from ATCC, were grown in OPTI-MEM (Gibco Laboratories, Grand Island, NY) supplemented with 5% FBS (culture medium). The cells were washed twice with serum-free medium before transfection with the TGF-β_1–chloramphenicol acetyl transferase (CAT) construct, plasmid phTG5, −453 to +11 nucleotide-sequence of human TGF-β_1 gene fused to the coding sequence of the bacterial CAT gene [8]. The transfected A-549 cells were washed and incubated overnight with medium alone, TGF-β_1, cyclosporine H (CsH) or CsA. TGF-β_1 promoter stimulation was quantified by measuring the concentration of CAT enzyme in extracts using a CAT-specific ELISA kit (Boehringer-Mannheim, Indianapolis, IN).

2.4. Isolation of nuclei and nuclear run off transcription assay

Nuclear run-on assay was performed according to a method described elsewhere [16]. Briefly, about 100–150 million T cells were lysed at 4°C by 0.02% NP-40 in a buffer containing 10 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 3 mM $CaCl_2$ and 0.3% sucrose. Nuclei were isolated by ultracentrifugation at 76,000 × *g* at 4°C for 45 min through a 2 M sucrose cushion in the same buffer. The nuclei were resuspended in the 1× run-on buffer containing 5 mM Tris-HCl, pH 7.5, 2.5 mM $MgCl_2$, 0.15 M KCl, 500 μM each of ATP, CTP, and GTP (Boehringer-Mannheim, Indianapolis, IN), 500 μCi [α-^{32}P]UTP (NEN DuPont), 2.5 μM DTT, 40 U of RNAsin (Promega, Madison, WI), 5 mM phosphocretanine (Sigma), 100 ng/ml creatinine kinase (Sigma, St. Louis, MO), and 10 μg/ml nucleotide diphosphate kinase (Sigma, St. Louis, MO). The reaction was carried out at 30°C for 30 min after which the reaction was stopped by adding 50 U of RNase-free DNase (Promega, Madison, WI) followed by another 5 min incubation. After the run-on reaction the radiolabeled RNA was isolated by using

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RNazol (Biorecx, Houston, TX), isopropanol precipitated, washed with 70% ethanol and dissolved in DEPC-treated water. An aliquot of radiolabeled RNA isolated from different T cell samples was analysed in duplicates for free vs. incorporated radioactivity by TCA precipitation and an equal amount of radiolabeled RNA (in terms of TCA precipitable radioactivity) from different treatment conditions were hybridized to alkali-denatured plasmid DNA (5 μg), immobilized on Zetaprobe (Bio-Rad, Richmond, CA) membrane using a slot blot apparatus (Bio-Rad, Richmond, CA). Hybridization was carried out in a total volume of 500 μl using 1–5 × 10^6 cpm/ml of hybridization solution (40% formamide, 4× SSC, 1× Denhardt's, 5 mM EDTA, 0.4% SDS and 100 μg/ml yeast tRNA at 42°C for 3 days. Hybridization was preceded by prehybridization of slot blot filters in the hybridization solution for 5–6 h. After hybridization, slot blot filters were first washed with 2× SSC, 0.1% SDS at room temperature for 30 min followed by three washes of 15 min each in 0.1× SSC, 0.1% SDS at 60°C and were autoradiographed for 3–7 days.

2.5. Preparation of nuclear extracts for electrophoretic mobility shift assay

The nuclear extracts were isolated as described [15]. In brief about 15 × 10^6 T cells were suspended in 100 μl buffer containing 100 mM Tris-HCl, pH 7.5, 2.5 mM $MgCl_2$, 3 mM $CaCl_2$, 3 mM DTT and 3% sucrose. Following this, NP-40 was added to the final concentration of 0.02% and the tube containing the cells was kept on ice for 5 min in a microfuge at 4°C. The supernatant was discarded and the nuclear pellet was resuspended in 100 μl of nuclear lysis buffer (20 mM HEPES, pH 7.9, 0.2 M KCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF and 20% glycerol). Nuclei were extracted for 1 h at 4°C. The nuclear extracts were then centrifuged at 100,000 × *g* for 30 min at 4°C. Aliquots of the nuclear extracts were frozen in liquid nitrogen and stored at −70°C. Protein concentration of the nuclear extracts were determined by the Bradford method [17].

2.6. Electrophoretic mobility shift assay

The binding reaction was carried out according to the method of Kim et al. [15] in a buffer containing 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA, 5% glycerol and 0.1 mM $ZnCl_2$ in a total volume of 20 μl, using 5–10 μg of protein in the nuclear extract and 0.2 ng of 5′ end-labeled double-stranded oligonucleotides in the presence of 1 μg of salmon sperm DNA at room temperature for 20 min. In some experiments, the nuclear extracts were first incubated with a rabbit antiserum reactive with the Sp1 protein or with a control rabbit serum. Protein–DNA complexes were resolved using a 5% polyacrylamide gel in 0.5× TBE, as described [12]. The gels were dried and exposed to X-ray films. The sequences of the different oligonucleotides (oligomers) used in this study were as follows: RCE, 5′-GGAGCCC-GCCCACGCGAGA-3′ [11,12]; Sp1, 5′-ATTCGATCGGGGCGGG-GCGAGA-3′ (Promega, Madison, WI); NF-κB, 5′-GGGATTTCAC-CT-3′ [15].

3. Results

3.1. Stimulation of TGF-β_1 promoter activity by CsA

A chimeric construct (CAT gene fused to a functional TGF-β_1 promoter [9]) and the A-549 cells were used to characterize the effect of CsA on promoter-dependent TGF-β_1 transcription. The transiently transfected A-549 cells were either untreated, or treated with CsA, CsH or TGF-β_1.

Fig. 1 illustrates the effect of CsA, CsH or TGF-β_1 on TGF-β_1 promoter activity. It is evident that CsA stimulates TGF-β_1 promoter-dependent transcription of the CAT gene. It is also evident that CsH, an inactive analogue of CsA [18], does not stimulate TGF-β_1-dependent transcription. Fig. 1 also shows that TGF-β_1, an inducer of TGF-β_1 expression [9], stimulates TGF-β_1 promoter activity.

3.2. Stimulation of TGF-β_1 transcription by CsA

Results from one of five nuclear run-on experiments are shown in Fig. 2 to illustrate the unique effect of CsA on TGF-β_1 gene transcription. CsA, in striking contrast to its effect on IL-2 transcription, stimulated TGF-β_1 gene transcription in T cells incubated with ionomycin and DAG (Fig. 2).

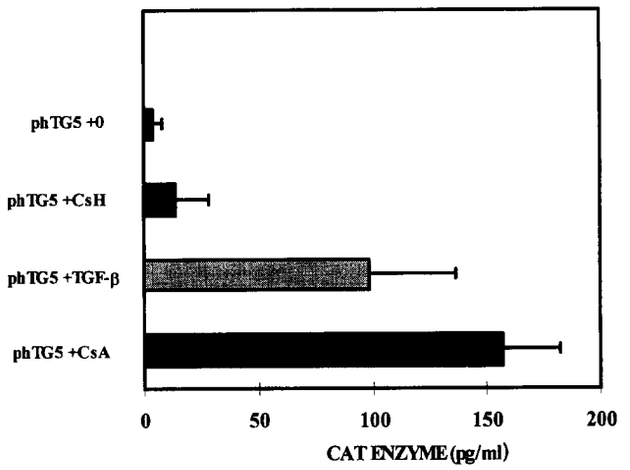

Fig. 1. Stimulation of TGF-β_1 promoter activity by CsA. A-549 cells, transiently transfected with the TGF-β_1 promoter–CAT gene chimeric construct, were treated with the reagents shown. TGF-β_1 promoter-driven CAT gene transcription was quantified by measuring CAT enzyme protein concentration with a CAT-specific ELISA. Results (mean ± S.E.M.) from 2–6 experiments (TGF-β_1 5 ng/ml; CsA and CsH 1 μg/ml).

3.3. Differential expression of RCF-1 and RCF-2 in T cells and lack of inhibitory activity by CsA

Transcription initiation is regulated by the binding of nuclear regulatory proteins to the enhancer/promoter region of a given gene. We therefore determined the effect of CsA on the emergence in T cells of DNA-binding proteins that bind to the

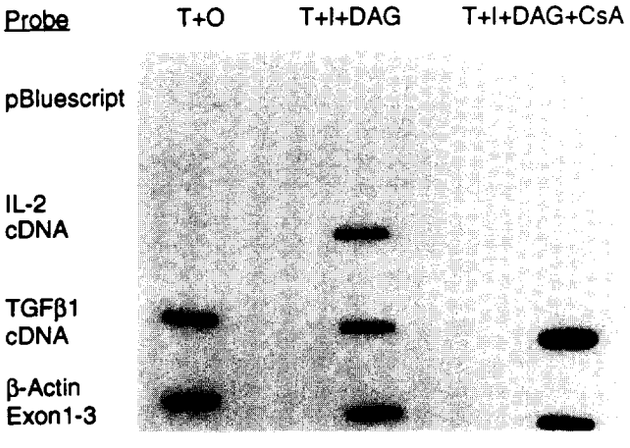

Fig. 2. TGF-β_1 gene transcription stimulation by CsA. T cells were incubated alone (T + 0), with ionomycin and DAG (T + I + DAG), or were pretreated with CsA and then incubated with ionomycin and DAG (T + I + DAG + CsA). The nuclei were isolated and the nuclear run-on transcription reaction was performed in the presence of α-^{32}P-labeled UTP. Whereas CsA pretreatment repressed the synthesis of IL-2 RNA transcripts, such pretreatment stimulated the synthesis of TGF-β_1 RNA transcripts. β-Actin gene transcription rate was quite similar in all three experimental conditions. This experiment is representative of five different experiments.

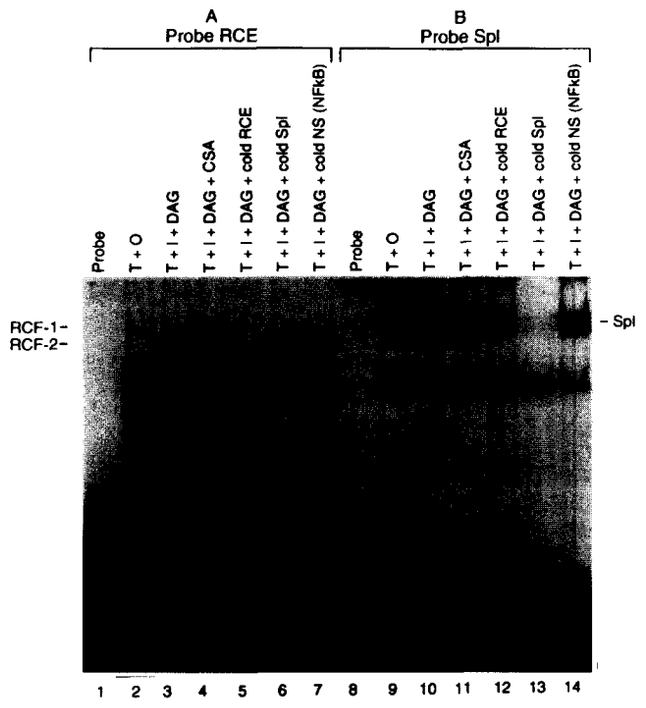

Fig. 3. Expression of RCF-1 and RCF-2 in T cells. Nuclear extracts, prepared from untreated or treated T cells, were incubated with α-^{32}P-labeled ds RCE (panel A) or Sp1 probe (panel B). (A) RCF-1 is present in equal amounts in the unstimulated T cells (lane 2), in T cells stimulated with ionomycin plus DAG (lane 3), and in T cells pretreated with CsA and then stimulated with ionomycin and DAG (lane 4). RCF-2 is absent in resting T cells (lane 2), emerges in the stimulated T cells (lane 3), and the emergence of RCF-2 is not inhibited by CsA (lane 4); RCF-1 and RCF-2 are out-competed by an excess of unlabeled ds RCE (lane 5). An excess of unlabeled ds Sp1 competes for RCF-1 only and not for RCF-2 (lane 6), and an excess of ds non-specific probe (NF-κB) does not compete for either RCF-1 or RCF-2 (lane 7). (B) Sp1 is present in equal amounts in resting T cells (lane 9), in stimulated T cells (lane 10) and in T cells pretreated with CsA and then stimulated (lane 11). Unlabeled excess ds RCE and the ds Sp1 compete for the Sp1 band (lanes 12, 13). An excess of non-specific probe NF-κB does not interfere with the formation of DNA–protein complexes.

promoter region of TGF-β_1. Specifically, the emergence of RCF-1 and RCF-2 in the T cells was evaluated since these DNA-binding proteins target RCE located in the promoter region of the TGF-β_1 gene and regulate TGF-β_1 transcription in cell lines [12]. The emergence of RCF-1 and RCF-2 in T cells was determined using ds DNA oligomers corresponding to the RCE sequence. Since RCF-1 appears to be Sp1, the emergence of nuclear protein(s) that bind the consensus Sp1 binding site was also determined.

Results found with the RCE probe are shown in Fig. 3A. RCF-1 was clearly evident in the nuclear extracts isolated from unstimulated T cells, appeared to be a doublet and was neither increased nor decreased in the nuclear extracts of T cells that were either untreated or pretreated with CsA, prior to stimulation with ionomycin plus DAG (panel A, lanes 2, 3, and 4). RCF-2 was not detected in the unstimulated T cells (lane 2) and was clearly induced in the T cells stimulated with ionomycin plus DAG (lane 3). It is also shown in Fig. 3 that CsA does not prevent the emergence of RCF-2 in the stimulated T cells (lane 3 vs. 4).

The specificity of DNA–protein complexes formed is demonstrated by the finding that the binding of either RCF-1 or RCF-2 to RCE is prevented by the unlabeled RCE probe (lane 5), and not by a non-specific probe (lane 7). That the RCF-1 detected in the nuclear extract of T cells is indeed Sp1 transcription factor is indicated by the finding that the formation of RCF-1–DNA complex is blocked by the unlabeled Sp1 probe (lane 6). Interestingly, Sp1 probe did not prevent RCF-2 binding to RCE (lane 6).

Results found with the Sp1 probe are illustrated in Fig. 3B. Sp1 was detected in unstimulated T cells (lane 9) and was not increased in T cells that were stimulated with ionomycin plus DAG (lane 10). Sp1 was also detected in T cells that were pretreated with CsA prior to stimulation (lane 11). The level of expression of Sp1 in CsA pretreated T cells was quite similar to that found in untreated cells and that found in T cells stimulated with ionomycin plus DAG (lanes 9–11).

The specificity of the binding reaction shown is demonstrated by the ability of the unlabeled specific Sp1 probe to inhibit DNA–protein complex formation (lane 13) and the inability of the unlabeled non-specific probe to prevent the formation of the complex between the nuclear protein and the Sp1 probe (lane 14). In Fig. 3 it is also shown that the unlabeled RCE probe reduces the binding of Sp1 protein to the Sp1 probe (Fig. 3B, lane 12).

4. Discussion

The new finding that has emerged from our study is that CsA, a well-characterized inhibitor of gene transcription, stimulates TGF-β_1 transcription in mammalian cells.

Two independent and complementary lines of evidence support the unique observation that CsA stimulates TGF-β_1 transcription. First, CsA stimulated TGF-β_1 promoter-dependent transcription of the CAT gene (Fig. 1). Second, CsA stimulated the synthesis of TGF-β_1 RNA transcripts in T cells incubated with a synergistic combination of DAG and ionomycin (Fig. 2).

A high degree of specificity regarding CsA was clearly evident in this study. CsA, and not CsH, an inactive analogue of CsA that has D-methylalanine instead of L-methylalanine at amino acid position 11, stimulated TGF-β_1 promoter activity (Fig. 1). Also, the stimulatory effect of CsA was gene specific. Whereas TGF-β_1 gene transcription was stimulated by CsA, IL-2 gene transcription was inhibited in the same T cell population (Fig. 2).

Studies exploring the mechanistic basis for the immunological activities of CsA have demonstrated that (i) cyclophilin, a ubiquitously distributed protein and a peptidyl-prolyl *cis-trans* isomerase, is a cellular receptor for CsA; (ii) CsA–cyclophilin complex binds and inactivates calcineurin, a calcium- and calmodulin-dependent serine-threonine phosphatase implicated in T cell signalling; and (iii) the emergence of NF-AT, a DNA binding protein and the induced expression of IL-2 gene are inhibited by CsA. Indeed, the ability of CsA to prevent IL-2 expression is currently considered to be the primary mechanism of action of CsA.

The NF-AT/IL-2 inhibition hypothesis [1,6], however, fails to offer a satisfactory explanation regarding the two well-docu-

mented complications of CsA: fibrosis and hypertension. Our demonstration that CsA stimulates TGF-β_1 transcription advances a novel mechanism not only for the immunosuppressive activity of CsA (since TGF-β_1 is a potent inhibitor of T cell activation) but also for fibrosis (since TGF-β_1 is a fibrogenic cytokine) and hypertension (since TGF-β_1 stimulates the production of endothelin-1, a potent vasoconstrictor polypeptide [19]).

Our studies on the emergence of DNA-binding proteins in primary T cells have also identified several novel features. First, CsA failed to inhibit activation-dependent emergence of RCF-2 in stimulated T cells (Fig. 3). This lack of inhibition is quite unique since CsA prevents the activation-dependent appearance of other DNA-binding proteins, like NF-AT [6,15], AP-1 or NF-κB in primary T cells [15]. Second, RCF-2, detected in our stimulated T cells, appears to be a nuclear binding protein that is distinct from Sp1 since the Sp1 oligonucleotide probe competed for the binding of RCF-1 protein and not for that of RCF-2 protein to TGF-β_1 RCE (Fig. 3).

The possibility that CsA promotes TGF-β_1 transcription by preventing the emergence of proteins that bind negative regulatory elements in the TGF-β_1 promoter is not excluded by our current study. This postulate is worthy of exploration given the precedent that CsA stimulates laccase gene transcription in the fungus *Cryphonectria parasitica* [20] by interfering with the negative regulator of lac-1 promoter activity.

In summary, results from this investigation demonstrate for the first time that CsA stimulates TGF-β_1 gene transcription in mammalian cells. This stimulation is associated with an inability of CsA to prevent the emergence of RCF-2, a known regulator of TGF-β_1 transcription.

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