

Differential Regulation of Transforming Growth Factor β and Interleukin 2 Genes in Human T Cells: Demonstration by Usage of Novel Competitor DNA Constructs in the Quantitative Polymerase Chain Reaction

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Summary

The regulation of mRNA encoding transforming growth factor β (TGF- β) and interleukin 2 (IL-2) in normal human T cells was explored using novel competitor DNA constructs in the quantitative polymerase chain reaction and accessory cell-independent T cell activation models. Our experimental design revealed the following: (a) TGF- β mRNA and IL-2 mRNA are regulated differentially in normal human T cells, quiescent or signaled with the synergistic combinations of: sn-1,2-dioctanoylglycerol and ionomycin or anti-CD3 monoclonal antibody (mAb) and anti-CD2 mAb; (b) the steady-state level of TGF- β mRNA in the stimulated T cells, in contrast to that of IL-2 mRNA, is increased by the immunosuppressant cyclosporine (CsA); and (c) the paradoxical effect of CsA on TGF- β mRNA levels is also appreciable at the level of production of functionally active TGF- β protein. Our findings, in addition to demonstrating the utility of the competitor DNA constructs for the precise quantification of immunoregulatory cytokines, suggest a novel and unifying mechanistic basis for the immunosuppression and some of the complications (e.g., renal fibrosis) associated with CsA usage.

Transforming growth factor β (TGF- β), a 25-kD homodimeric multifunctional cytokine and a secretory product of many cell types including T cells, is a potent inhibitor of T cell growth and differentiation (1-3). IL-2, on the other hand, promotes T cell growth and their acquisition of specialized effector functions (4). TGF- β and IL-2, thus, have the potential to exert diametrically opposite effects on the expression of antigen-specific T cell immunity. It was of interest, therefore, to quantify, simultaneously, the regulation of mRNA encoding TGF- β and IL-2 in normal human T cells. Also, in view of the ability of TGF- β to inhibit the antiallograft response (5), it was considered important to quantify the effect of cyclosporine (CsA) on the induction of TGF- β mRNA in normal human T cells, and compare it to its well-characterized inhibitory effect on the induction of IL-2 mRNA (6).

Materials and Methods

Isolation and Activation of T Cells. T cells were isolated from normal human PBMC with a sequential multi-step procedure that yields >98% CD2 antigen-positive cells and <1% cells that are

positive for the DR, CD14, CD25, or CD56 antigens (7). Accessory cell-independent T cell activation was accomplished with either sn-1,2-dioctanoylglycerol (DAG; 10.0 μ g/ml) and ionomycin (1.0 μ M) or crosslinked anti-CD2 (OKT11; 0.5 μ g/ml) and anti-CD3 (OKT3; 0.5 μ g/ml) mAbs (7).

Design and Synthesis of Competitor DNA Constructs. Fig. 1 illustrates the design, synthesis, and authentication of the 290-bp TGF- β competitor DNA construct created for the quantification of TGF- β mRNA by PCR. As shown, the oligonucleotide primer pair was designed to amplify a region in the TGF- β gene that contains a MseI restriction site. The MseI digestion of the 246-bp TGF- β PCR product yielded 210- and 36-bp subfragments that were annealed with a 44-bp DNA insert synthesized in vitro to have cohesive ends for the MseI restriction site at 5' and 3' ends. The phosphorylated 44-bp DNA fragment was ligated with the 210- and 36-bp DNA fragments using *Escherichia coli* DNA ligase. After ligation, the mixture was run on a 2% low melting point agarose gel, and the 290-bp fragment was eluted and purified using the gene clean kit.

The 178-bp IL-2 competitor DNA construct was created in a similar fashion. Here, the phosphorylated 44-bp DNA insert was ligated with the 88- and 46-bp subfragments derived by MseI digestion of the IL-2 PCR product.

Quantification by Competitive PCR. The cDNA, for quanti-

fication by competitive PCR (8), was synthesized in a reverse transcription reaction mixture containing 1 μ g of total RNA (isolated from normal human T cells by the guanidinium isothiocyanate/cesium chloride method), 100 ng of random hexanucleotide primers, and 200 U of moloney murine leukemia virus reverse transcriptase. A constant amount of the cDNA (2 μ l) was then coamplified with known concentrations of the competitor DNA construct, for 32 cycles using 200 μ M TGF- β or IL-2 gene-specific primer pairs in a reaction mixture (50 μ l) containing 1 \times Taq buffer, 1 U Taq DNA polymerase, and 40 μ M of each dNTP. The PCR products were resolved by 2% agarose gel electrophoresis, visualized by ethidium bromide staining, and photographed. The negative of the photographs was analyzed by laser densitometry, and the absolute absorbance values of the PCR products were determined. The ratios of the absorbance of the relevant PCR product pairs (290-bp TGF- β competitor and 246-bp TGF- β ; 178-bp IL-2 competitor and 149-bp IL-2 PCR product) were plotted against the concentration of the competitor DNA used. The concentration of mRNA was thus determined and expressed as attomoles (amol) of TGF- β or IL-2 mRNA/mg of total RNA.

Results and Discussion

Differential Regulation of TGF- β mRNA and IL-2 mRNA. The steady-state levels of TGF- β mRNA and IL-2 mRNA in normal human T cells, quantified by competitive PCR, are displayed in Table 1. Several features that distinguish TGF- β mRNA regulation in T cells from that of IL-2 mRNA are apparent. First, in quiescent normal human T cells, TGF- β mRNA is readily quantifiable at every time point tested, and IL-2 mRNA is not detectable, at any of the time points evaluated, even by the application of the sensitive PCR methodology (Table 1). Second, quantitative and qualitative differences exist between TGF- β mRNA and IL-2 mRNA regulation in T cells signaled with the synergistic combination of DAG and ionomycin. As shown in Table 1, the concentration of IL-2 mRNA in the stimulated T cells is several-fold greater than that of TGF- β mRNA in the same T cell

population, and the highest concentration of IL-2 mRNA is found 4 h after stimulation, and that of TGF- β mRNA 1 h after signaling of T cells with DAG and ionomycin.

The most striking difference between TGF- β mRNA regulation and that of IL-2 mRNA was evident when T cells, pretreated with CsA, were signaled with DAG and ionomycin. Whereas CsA completely prevented activation-dependent IL-2 mRNA accumulation in the T cells, the steady-state levels of TGF- β mRNA were increased by CsA (Table 1). The CsA-mediated increase in the abundance of TGF- β mRNA was best seen at the 1 h time point (Table 1).

Demonstration of Differential Regulation of TGF- β mRNA and IL-2 mRNA by Crosslinking the CD3 and CD2 Antigen on the T Cell Surface. Differential regulation of TGF- β mRNA and IL-2 mRNA, uncovered in this instance, by transmembrane signaling of T cells by crosslinkage of the CD3/TCR complex with the CD2 antigen is illustrated in Fig. 2. Laser densitometric scanning of the agarose gel electrophoresis analyses data displayed in Fig. 2 and its computation resulted in the following values (per mg of RNA from T cells): unstimulated T cells, 1,038 amol of TGF- β mRNA and undetectable level of IL-2 mRNA (Fig. 2, A1 and B1); T cells stimulated with crosslinked anti-CD3 and anti-CD2 mAbs, 1,992 amol of TGF- β mRNA and 17,170 amol of IL-2 mRNA (Fig. 2, A2 and B2); and T cells stimulated in the presence of CsA, 9,903 amol of TGF- β mRNA and undetectable level of IL-2 mRNA (Fig. 2, A3 and B3). Thus, CsA increased the steady-state level of TGF- β mRNA from 1,992 to 9,903 amol in T cells stimulated via cell surface proteins implicated in physiological antigenic signaling.

CsA Enhances TGF- β Protein Production. CsA's ability to augment TGF- β gene expression was evident at the level of production of functionally active TGF- β protein. As illustrated in Fig. 3, the highest levels of TGF- β protein were found in the supernatants of T cells pretreated with CsA and then signaled with either DAG and ionomycin or with cross-

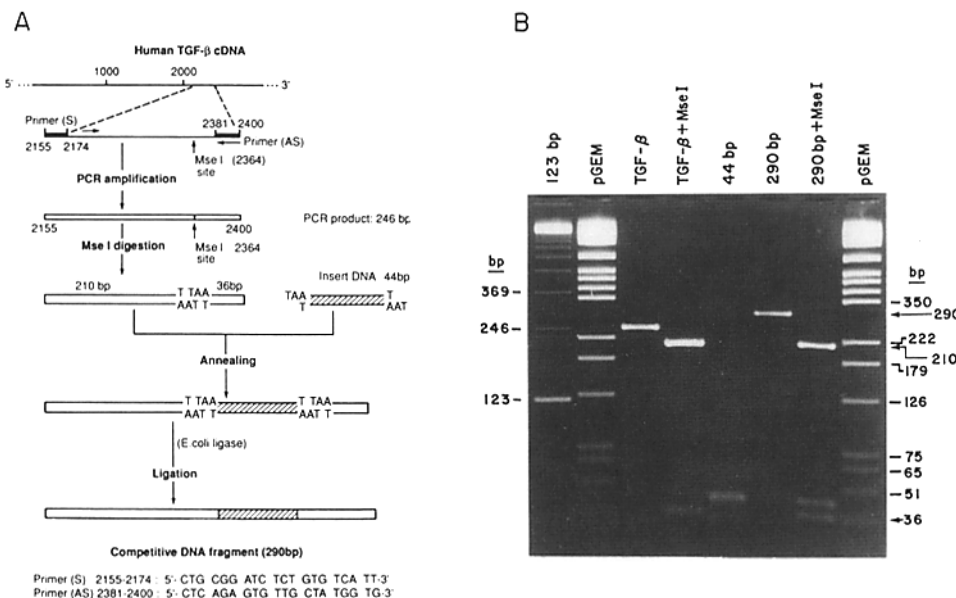


Figure 1. Design and authentication of the TGF- β competitor DNA construct. The primer design, the restriction site for MseI, the subfragments generated by MseI digestion of the TGF- β PCR product, the insert DNA construct are all schematically shown in A. Agarose gel electrophoretic validation of the 246-bp TGF- β PCR product, generation of the appropriate sized subfragments of 210 and 36 bp lengths after digestion with MseI, the insert DNA of 44 bp, and the 290-bp competitor DNA construct are shown in B. The digestion with MseI of the 290-bp TGF- β competitor DNA construct yielding subfragments of 210, 44 and 36 bp and the 123-bp and the pGEM ladders are also shown in B.

Table 1. Differential Regulation of TGF- β mRNA and IL-2 mRNA in T Cells

Exp.	Additions to T cells*	Amount of mRNA in T cells							
		1 h		4 h		16 h		40 h	
		TGF- β	IL-2	TGF- β	IL-2	TGF- β	IL-2	TGF- β	IL-2
<i>attomol/mg of RNA</i>									
1	None	657	<20	406	<20	228	<20	200	<20
	DAG + Iono	2,408	2,736	441	24,419	172	2189	147	21
	CsA + DAG + Iono	5,488	<20	744	<20	238	<20	213	<20
2	None	360	<20	441	<20	431	<20	294	<20
	DAG + Iono	625	3,449	253	37,832	544	366	272	57
	CsA + DAG + Iono	1,567	<20	685	<20	272	<20	413	<20
3	None	431	<20	463	<20	156	<20	147	<20
	DAG + Iono	653	129	259	192,050	147	232	169	78
	CsA + DAG + Iono	1,085	<20	222	<20	181	<20	131	<20

* Highly purified T cells (10^6 cells/ml) were incubated with the agents shown for 1, 4, 16, or 40 h. Total RNA was then isolated, reverse transcribed into cDNA, and amplified by PCR. The amount of TGF- β mRNA was quantified by using the 290-bp TGF- β competitor DNA construct and the IL-2 mRNA with the 178-bp IL-2 competitor DNA construct in the competitive PCR. DAG, 10.0 μ g/ml; ionomycin, 1.0 μ M; CsA, 100 ng/ml.

linked anti-CD2 and anti-CD3 mAbs. The bioactivity (growth inhibition of mink lung epithelial cells) was best observed after acidification of the T cell supernatants and was inhibited, in each instance, by an avian anti-TGF- β antibody (Fig. 3 B).

Conclusions and Implications. TGF- β , a potent inhibitor of T cell growth and differentiation, is considered also as a fibrogenic cytokine (1, 2). Our observation that CsA enhances TGF- β gene expression, thus, suggests a novel and unifying mechanism for the immunosuppression as well as for the renal

fibrosis encountered with CsA usage. Our investigation also suggests a new hypothesis for the CsA-mediated inhibition of new DNA synthesis by T cells (since TGF- β inhibits *c-myc* gene expression and *c-myc* expression contributes to T-cell growth [9]) and for the CsA-associated vasoconstriction and hypertension (since TGF- β enhances the expression of the potent vasoconstrictor, endothelin [10]).

CsA has selective inhibitory effects on the appearance of DNA regulatory proteins (11). Thus, prevention by CsA of

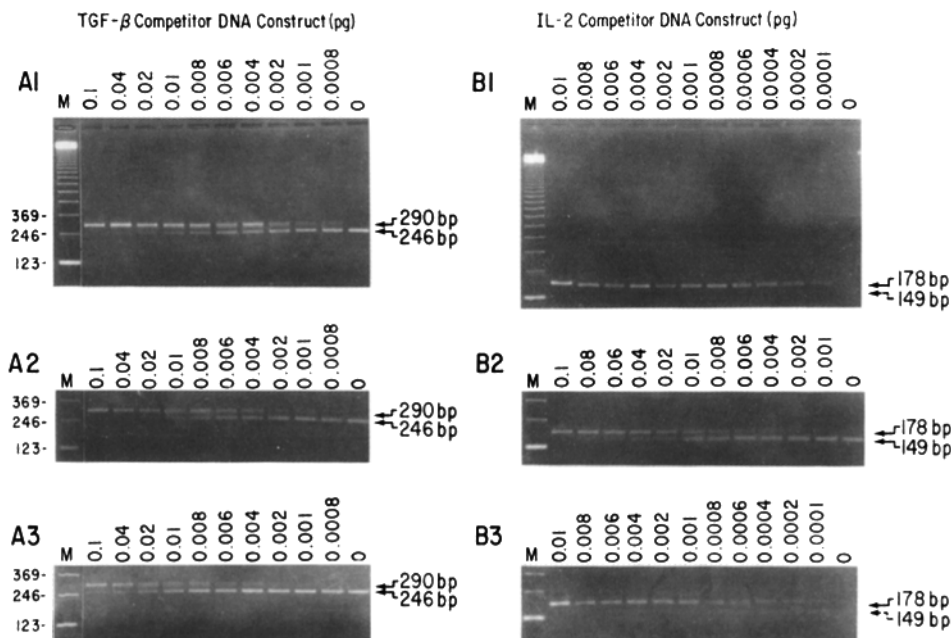


Figure 2. Differential regulation of TGF- β and IL-2 mRNA in T cells. Serial dilutions of the 290-bp TGF- β construct or the 178-bp IL-2 construct were coamplified with the cDNA from unstimulated T cells (A1 and B1), T cells stimulated with anti-CD2 mAb and anti-CD3 mAb (A2 and B2), or T cells pretreated with CsA (100 ng/ml) and stimulated with anti-CD2 mAb and anti-CD3 mAb (A3 and B3). The mRNA levels, found after 1 h of incubation of T cells, are shown and are provided in the text. M, 123-bp repeat ladder.

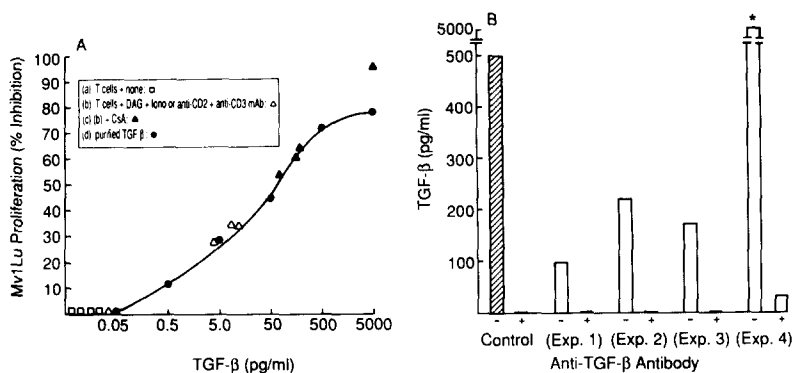


Figure 3. Augmentation of TGF- β protein production by CsA. (A) TGF- β protein concentration of T cell supernatants from four consecutive experiments. TGF- β bioactivity in the supernatants obtained after 40 h of incubation of T cells was quantified with the mink lung epithelial cell assay. (B) The effect of 12.5 μ g of neutralizing anti-TGF- β antibody on purified 500 pg/ml TGF- β (control) or on the TGF- β bioactivity found in the supernatants of T cells stimulated in the presence of 100 ng/ml of CsA.

the emergence of nuclear regulatory factors that bind to the negative regulatory sequence of the TGF- β gene or the lack of inhibition by CsA of DNA regulatory proteins that promote TGF- β gene expression might be responsible for our

results. These hypotheses as well as whether CsA differentially regulates TGF- β and IL-2 gene expression in vivo are to be explored in our future studies.

We thank Dr. Vijay K. Sharma for expert technical help, Dr. Kirk Manogue for careful reading of the manuscript and useful suggestions, Dr. Albert L. Rubin for encouragement, and Ms. Lorraine Goldberg Garcia for the meticulous secretarial help in the preparation of this manuscript.

This work was supported in part by U.S. Public Health Service grant AI-26932 (M. Suthanthiran) and by The American Cancer Society grant CN-30 (A. Cerami).

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Received for publication 3 July 1991 and in revised form 30 August 1991.

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