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The immunosuppressive agent tacrolimus induces p21WAF/CIP1WAF1/CIP1 via TGF-β secretion

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Abstract

Tacrolimus (Tac) is more immunosuppressive drug compared to cyclosporine (CsA). Our previous studies have demonstrated that CsA induces the expression of p21WAF/CIP1 expression. In this study we explored if like CsA, Tac also induces expression of p21WAF/CIP1. We also determined if induction of p21WAF/CIP1 by Tac is dependent on TGF-β. Using RT-PCR and Western blot analysis, we studied the induction of p21WAF/CIP1 mRNA and protein in human T cells and A-549 cells (human lung adenocarcinoma cells) by Tac. The stimulation of p21WAF/CIP1 promoter activity was studied by luciferase assay using p21WAF/CIP1-luc, chimeric plasmid DNA containing a p21WAF/CIP1 promoter segment and luciferase reporter gene. Using anti-TGF-β antibody, we studied if induction of p21WAF/CIP1 by tacrolimus is dependent on TGF-β. The results demonstrate that Tac induced p21WAF/CIP1 mRNA and protein expression as well as stimulated its promoter activity in T cells and A-549 cells. The induction of p21WAF/CIP1 expression by tacrolimus was dependent on TGF-β since a neutralizing anti-TGF-β antibody inhibited induction of p21WAF/CIP1in A-549 cells. These data support the hypothesis that cyclin inhibitor p21WAF/CIP1 might represent a unified mediator of the anti-proliferative effects of Tac and other immunosuppressive agents. Strategies involving p21WAF/CIP1 induction should be considered a viable alternative strategy to achieve immunosuppression possibly with reduced toxicity associated with current immunosuppression.

Keywords: Tacrolimus; p21; Immunosuppression; mRNA expression; Promoter activity; Western blot; Cyclins; Allo-immune activation

The mechanism(s) of the efficacy of tacrolimus has traditionally been explained on the basis of inhibition of IL-2 [1,2] and, more recently, through the induction of TGF-β [3,4]. Both processes involve the inhibition of cell cycle progression. The cell cycle is controlled by cyclins, regulated by their association with cyclin-dependent kinases (CDKs) which promote cell division [5,6]. Selective CDK inhibitors [7] capable of preventing the cells from entering the cell cycle inhibit CDKs. The expression of IL-2 mRNA and protein is increased in activated T cells, allowing them to enter cell cycle [8]. Studies have demonstrated that expression of a number of cyclins and cyclin-dependent kinases is elevated during the T-cell activation [9,10]. The treatment of tacrolimus prior to the stimulation of T-cell activation results in the inhibition of IL-2 gene expression and IL-2 protein [11]. We have previously reported that CsA and Tac induce the expression of TGF-β in lymphoid and non-lymphoid cells [3,4]. The studies have also demonstrated that TGF-β induces the cell cyclin inhibitor p21WAF/CIP1 [12], and the anti-proliferative effects of TGF-β appear to at least be associated with its induction p21WAF/CIP1 [13]. Our studies have also demonstrated that cyclosporine induces mRNA and protein expression of p21WAF/CIP1 and stimulates its promoter activity [14] which was dependent on TGF-β expression. Our studies have also demonstrated that p21WAF/CIP1 over-expression in lymphocytes renders them more responsive to the inhibitory effects of CsA and are less responsive to mitogenic stimuli [15]. From these results it can be concluded that major component of the immunosuppressive drug, cyclosporine, can be via p21WAF/CIP1 induction of CsA. Since CsA and tacrolimus share

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similar immunosuppressive and fibrogenic properties, accordingly, this study was designed to investigate whether like CsA, Tac induced p21WAF/CIP1 and whether this induction was dependent on TGF-β. We carried out these studies in purified human T cells and the human lung adenocarcinoma cell line A-549.

Materials and methods

Preparation of T cells. Highly purified T cells (>98% CD2 antigen positive cells) were isolated from normal human peripheral blood mononuclear cells as described [4]. In brief, peripheral blood mononuclear cells from healthy donors were separated by ficoll-hypaque and incubated in 75-cm^2 flasks overnight [RPMI containing 5% fetal bovine serum (FBS) and supplemented with penicillin–streptomycin] to remove the adherent cell population. The cells were then incubated on a sephadex G-10 column, eluted with RPMI containing 20% FBS, washed with PBS, counted and mixed with 5% sheep red blood cells (SRBCs) overnight, and incubated at 4 °C. T-cell rosettes, obtained after centrifugation on ficoll-hypaque gradient were pooled, SRBCs were lysed, and T cells were obtained after centrifugation. The purity of T cells was ascertained by staining with T-cell surface antigen specific antibodies using single cell analysis by flow cytometry. Purified T cells were activated with a synergistic combination of Phorbol 12-Myristate 13-acetate (PMA 10 ng/ml) and ionomycin (400 ng/ml).

To study the effect of tacrolimus resting T cells were pretreated with 10 ng/ml of tacrolimus or similar concentration of ethanol diluted in PBS as a vehicle control for tacrolimus, 30 min prior to activation. Cells were harvested after 4 h of activation, RNA was isolated, and p21WAF/CIP1mRNA expression was studied by RT-PCR.

Preparation of A-549 cells. We used A-549 cells, a human lung adenocarcinoma cell line, to study the induction of p21WAF/CIP1 by tacrolimus. The treatment of these cells with CsA and tacrolimus results in their growth arrest and increased TGF-β protein secretion [3,4]. A-549 cells were grown in OPTI-MEM (Gibco, Long Island, NY) containing 5% fetal bovine serum at 37 °C in a 5% CO_2 and 95% air atmosphere. After 72 h of culture, cells were trypsinized, washed with medium, and counted as described [3]. The cells, washed twice with serum-free medium, were incubated with and without tacrolimus (125–500 ng/ml). Cells were used to isolate RNA to semi-quantify p21WAF/CIP1 mRNA by RT-PCR and also to prepare lysates for Western blot analysis of p21WAF/CIP1 protein expression.

Gene expression by PCR. Total RNA was isolated from T lymphocytes and A-549 cells using Trizol isolation System (Life Sciences, Invitrogen) and quality of RNA was verified by 260/280-nm ratio. One microgram of RNA was reverse-transcribed to cDNA using Superscript First Strand Synthesis system for RT-PCR (Life Technologies, Rockville MD). The amplification by polymerase chain reaction (PCR) was carried out using 1 μl cDNA and 2 μl each of 2.5 mM coding and non-coding oligonucleotide primers and Platinum PCR Supermix (Life Technologies, Rockville, MD). The primer sequences used were; p21WAF/CIP1: coding 5′-AGG ATC CAT GTC AGA ACC GGC TGG-3′; noncoding 5′-CAG GAT CCT GTG GGC GGA TTA GGG-3′ [16]. β-actin: coding 5′-TGA CGG GGT CAC CCA CAC TGT GAA CAT CTA-3′; noncoding 5′-CTT GAA GCA TTT GCG GTG GAC GAT GGA GGG-3′ [17]. The PCR amplification profile consisted of 95 °C for 45 s, 60 °C for 45 s, and 72 °C for 75 s. Amplification for p21WAF/CIP1 and β-actin was carried out for 33 and 27 cycles, respectively. The PCR products were resolved in 1% agarose gel electrophoresis and ethidium bromide stained specific bands were visualized under UV light and photographed. The densitometric analysis of the specific bands was made using Alpha-Imager (Alpha Innotech, San Leandro, CA) and data are represented as the ratio of the specific gene to β-actin.

Effect of tacrolimus on the promoter activity of p21WAF/CIP1 gene. Promoter activity was studied as previously described [14]. A-549 cells were transfected with p21WAF/CIP1-luc plasmid DNA (a segment of p21WAF/CIP1 promoter region and luciferase gene). The wild-type p21WAF/CIP1 promoter used was a segment of p21WAF/CIP1 promoter, which contained a TGF-β response element (TβRE). According to Datto et al. [12] based on the electromobility shift assays, this segment also contains the consensus sp1 binding sites which are responsible for the induction of p21WAF/CIP1 expression by TGF-β. A-549 cells were cultured overnight in 6-well plates, cells were washed with serum-free medium and transfected with 1 μg DNA complexed with 5 μl of lipofectamine (Gibco, Long Island, NY) for 5 h, and the cells were then incubated with opti-MEM containing 5% FBS. The cells were washed with serum-free medium and treated with tacrolimus (125 ng/ml) for 24 h. The cells were washed with PBS and cell lysates were prepared using lysis buffer (Promega, Madisin, WI). The luciferase activity was quantified in the cell lysates. Induction of the promoter activity by tacrolimus was measured relative to the activity without tacrolimus. Since we have earlier reported that CsA stimulates promoter activity of p21WAF/CIP1, CsA (250 ng/ml) was used as a positive control in each experiment.

Induction of p21WAF/CIP1 protein expression by tacrolimus. The expression of p21WAF/CIP1 protein in A-549 cells treated with different concentrations of tacrolimus was studied by Western blot analysis of cell lysates. A-549 cells were grown overnight in 6-well plates and cells were washed with serum-free medium and incubated with CsA (125, 250 ng/ml) for 4 h. The cells were washed 2× with PBS and lysed with pre-heated (100 °C) lysis buffer (10 mM Tris–HCl, 1% SDS, and sodium vanadate 200 μM). The protein was quantified using Bio-Rad (Hercules, CA) Protein Assay Kit. An equal amount of protein (20 μg) was electrophoresed for each sample. The expression of

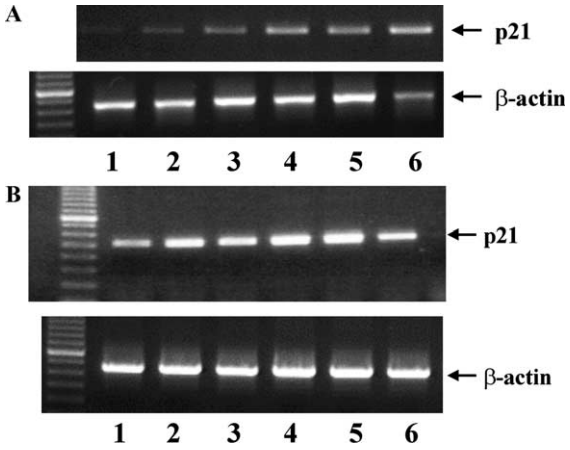

Fig. 1. Effect of tacrolimus on p21WAF/CIP1 expression in A-549 cells: (A) Effects of different concentrations of tacrolimus: Effects of different concentrations of tacrolimus on p21WAF/CIP1 mRNA expression are shown in the figure. A-549 cells were untreated (lane 1); treated with tacrolimus 12.5 ng/ml (lane 2); 25 ng/ml (lane 3); 50 ng/ml (lane 4); 100 ng/ml (lane 5); and 200 ng/ml (lane 6). (B) Effect of tacrolimus on p21WAF/CIP1 mRNA in A-549 cells at different time intervals. We also conducted a number of experiments to work on the time course of the increase in p21WAF/CIP1 mRNA expression after treatment with 50 ng/ml of tacrolimus. The effect of tacrolimus on p21WAF/CIP1 mRNA in A-549 cells at different time intervals 0 (lane 1), 30 min (lane 2), 1 h (lane 3), 2 h (lane 4), 3 (lane 5), and 4 h (lane 6) is shown.

p21WAF/CIP1 was analyzed using an anti-p21WAF/CIP1monoclonal antibody and compared with control p21WAF/CIP1 protein (Transduction Labs, TX, USA).

Results

Effect of different concentrations of tacrolimus on p21WAF/CIP1 mRNA in A-549 cells

Dose-dependent p21WAF/CIP1 induction by tacrolimus

Representative figure (Fig. 1A) of four consecutive experiments show a dose-dependent increase of p21WAF/CIP1 mRNA expression in A-549 cells. Increased p21WAF/CIP1 mRNA expression can be seen with as low as 12.5 ng/ml of tacrolimus, a therapeutic concentration in transplant patients treated with tacrolimus.

Time-course of the effect of tacrolimus on p21WAF/CIP1 mRNA in A-549 cells

Results from a representative of four consecutive experiments are shown in Fig. 1B. A sharp increase was observed at 2-h post treatment with tacrolimus (50 ng/ml), which continued until 3 h with a decrease at 4-h post-tacrolimus treatment. Even at this time point ratio of p21WAF/CIP1 with β-actin was higher than that of untreated cells (data not shown).

Effect of tacrolimus on p21WAF/CIP1 mRNA in T cells

To study the effect of tacrolimus on p21WAF/CIP1 mRNA in T cells, cells were treated with tacrolimus (10 ng/ml) prior to activation with PMA/ionomycin. The results from four consecutive experiments are shown in Fig. 2. The expression of p21WAF/CIP1 mRNA was significantly ($p < 0.05$) increased in T cells activated in the presence of tacrolimus.

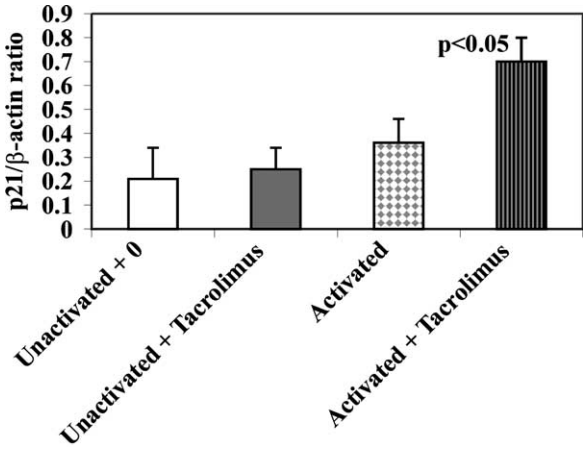

Fig. 2. Effect of tacrolimus on p21WAF/CIP1 mRNA expression in T cells: The expression of p21WAF/CIP1 mRNA in T cells at 4 h post activation is shown. *Y*-axis represents the p21WAF/CIP1 gene/β-actin ratio. In contrast to the unctivated T cells with and without tacrolimus (10 ng/ml), a significant ($p < 0.01$) increase in p21WAF/CIP1 mRNA at 4 h can be seen in T cells activated with tacrolimus ($n = 4$ experiments).

Whether p21WAF/CIP1 induction by tacrolimus like CSA is also dependent on TGF-β

In these experiments we tested if Tac induced p21WAF/CIP1 is dependent on TGF-β, to study the induction of p21WAF/CIP1 gene and protein expression in A-549 cells (human lung adenocarcinoma cell line) inhibited by TGF-β both in the presence and the absence of anti-TGF-β or control antibody. Untreated cells and cells treated with anti-TGF-β antibody alone were used as controls. Fig. 3A demonstrates the effect of anti-TGF-β antibody on p21WAF/CIP1 mRNA expression in A-549 cells. The concentration of TGF-β antibody

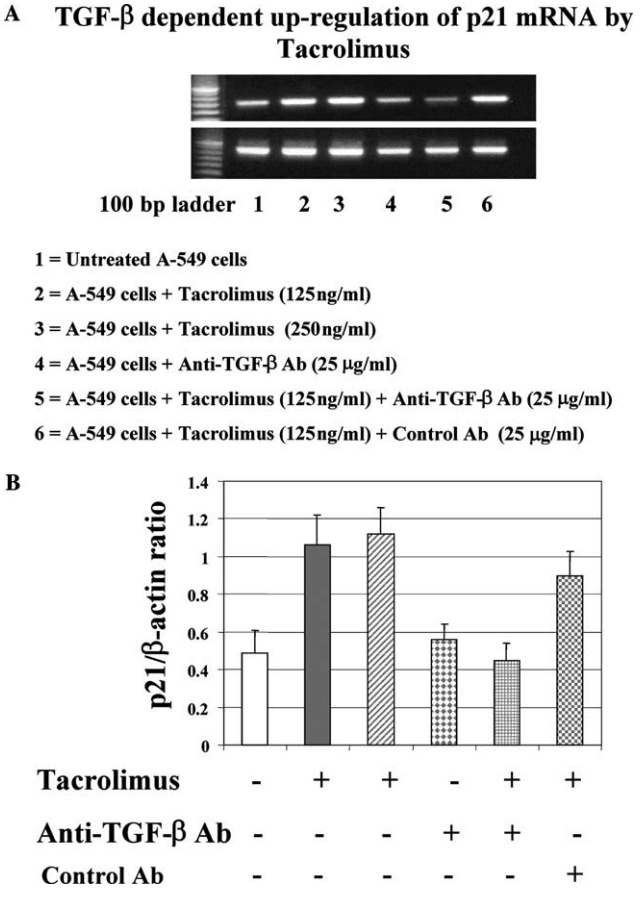

Fig. 3. Effect of anti-TGF-β antibody on the induction of p21WAF/CIP1 mRNA. Experiments were conducted with tacrolimus treated A-549 cells cultured with or without anti-TGF-β antibody. (A) A representative experiment showing the effect of anti-TGF-β antibody on tacrolimus induced p21WAF/CIP1 mRNA expression. Anti-TGF-β antibody alone and tacrolimus alone are also shown. (B) Mean ± SEM of the ratio of the densitometric analysis of for p21 WAF/CIP1 with β-actin mRNA is shown, demonstrating that anti-TGF-β antibody reversed tacrolimus-induced p21WAF/CIP1 mRNA expression ($n = 4$, untreated vs. tacrolimus, $p < 0.02$ and tacrolimus (125 ng/ml) vs tacrolimus plus anti-TGF-β antibody <0.05).

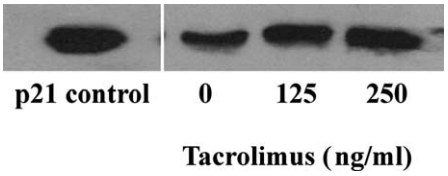

Fig. 4. Tacrolimus induces increased expression of p21WAF/CIP1 protein in A-549 cells. The expression of p21WAF/CIP1 protein in A-549 cells treated with different concentrations of tacrolimus was studied by Western blot analysis of cell lysates. The expression of p21WAF/CIP1 was analyzed using an anti-p21WAF/CIP1monoclonal antibody (Transduction labs, TX, USA). p21WAF/CIP1 protein is increased using both concentrations of tacrolimus. Control positive for p21WAF/CIP1 protein is also shown.

used was 25 μg/ml. The TGF-β antibody but not the control antibody could significantly inhibit the expression of p21WAF/CIP1 mRNA in A-549 cells treated with tacrolimus 125 ng/ml. The composite data from four such experiments presented as the ratio of densitometric numbers obtained for ethidium bromide stained bands of p21WAF/CIP1 and β-actin are shown in Fig. 3B. These results demonstrate that the treatment of A-549 cells results in the increased expression of p21WAF/CIP1 mRNA whereas anti-TGF-β antibody inhibits the expression of p21WAF/CIP1 mRNA induced by tacrolimus. We also studied the effect of tacrolimus on TGF-β protein in the conditioned medium. A more than four-fold increase ($p < 0.02$) TGF-β protein was observed in tacrolimus-treated A-549 cells compared to untreated cells (92 ± 23 vs. 365 ± 88 pg/ml).

Effect of tacrolimus on p21WAF/CIP1 protein expression

To further confirm and authenticate these results, we also studied the expression of p21WAF/CIP1 protein expression by Western blot analysis. The results shown in Fig. 4 demonstrate that tacrolimus induces p21WAF/CIP1 protein expression in A-549 cells.

Effects of tacrolimus on promoter activity of p21WAF/CIP1 gene in A-549 cells

In order to understand the mechanism of the induction of p21WAF/CIP1 protein and mRNA expression by tacrolimus, the effect of tacrolimus on the promoter activity of p21WAF/CIP1 gene was studied in A-549 cells. After the transfection with a plasmid DNA containing a fragment of p21WAF/CIP1 promoter and luciferase, the reporter gene, the cells were cultured for 24 h with or without tacrolimus. Since we speculated that tacrolimus induced TGF-β protein may mediate increase in p21WAF/CIP1's promoter activity, we treated the cells with tacrolimus for 24 h at a time point where maximal TGF-β protein is secreted. Since we have already published the effect of CsA on p21WAF/CIP1 promoter, we used CsA as positive control in these experiments. The lysates were prepared and the luciferase activity was quantified using a luminometer and expressed as relative light units. Fig. 5 demonstrates the statistically significant increased promoter activity by tacrolimus ($p < 0.0001$) and CsA ($p < 0.02$) in A-549 cells transfected with a plasmid construct reported to be TGF-β inducible due to a TGF-β response element. These results demonstrate that like cyclosporine A, tacrolimus also stimulates significantly more promoter activity of p21WAF/CIP1 gene.

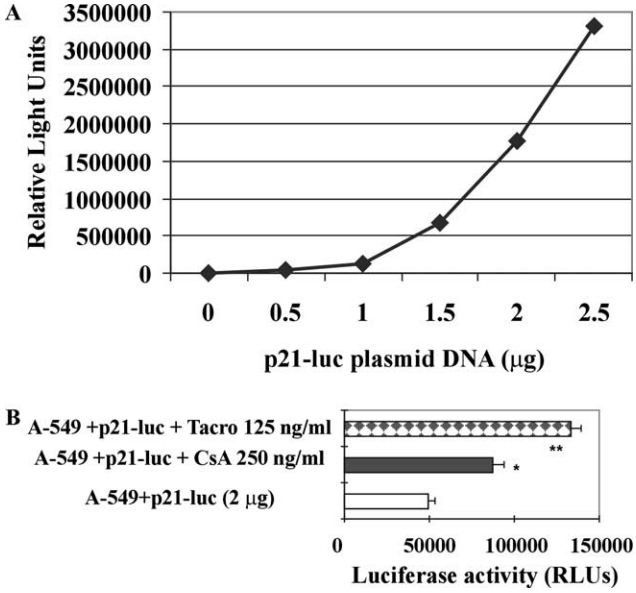

Fig. 5. Tacrolimus stimulates the promoter activity of p21WAF/CIP1 gene in A-549 cells. (A) Effect of tacrolimus (125 ng/ml) on promoter activity in A-549 cells transfected with various concentrations of p21WAF/CIP1-luc plasmid DNA is shown. A dose-dependent effect can be seen. (B) The mean and SEM of the relative light units from four consecutive experiments shows the statistically significant increase in promoter activity induced by tacrolimus, $^{**}p < 0.0001$. Effect of CsA was studied as positive control, which significantly stimulated promoter activity of p21WAF/CIP1 A in A-549 cells, $^{*}p < 0.02$.

Discussion

The data presented here demonstrate that tacrolimus stimulates the expression of p21WAF/CIP1 mRNA, in both T lymphocytes and non-lymphoid cells (A-549 cells), which is dependent on TGF-β. Tacrolimus also stimulates its promoter activity and protein expression. Our previous studies [3,4,11] demonstrated that the current immunosuppressive drugs; cyclosporine, tacrolimus, and rapamycin induce expression of TGF-β besides inhibiting IL-2 expression. TGF-β is one of the most potent immunosuppressive molecules and inducer of fibrogenesis [18,19]. The cyclin inhibitor p21WAF/CIP1, which is a potent inhibitor of cellular proliferation, mediates the inhibitory effects of TGF-β [12,13]. Our earlier studies [14] also demonstrated that CsA, the

mostly commonly used primary immunosuppressant in organ transplantation, induces the expression of p21 WAF/CIP1. Also, in vitro and in vivo over-expression of p21WAF/CIP1 results in decreased lymphocyte proliferation and increased susceptibility of lymphocytes to the inhibitory effects of cyclosporine [15]. p21WAF/CIP1 is one of the most potent regulators of the cell-cycle and is known to inhibit cell proliferation by two independent and functionally different ways. It binds to Cdk2, also binds and inhibits PCNA (proliferating cell nuclear antigen) which is an auxiliary protein in DNA polymerase needed for DNA synthesis and nucleotide excision repair [20], and has six binding sites for p21WAF/CIP1 [21].

During this process of T-cell activation a number of cyclins and cyclin-dependent kinases are hyper-expressed and activated [9,10]. Sutherland et al. [22] studied the effects of cyclosporine A on splenocytes before and after exposure to recombinant interleukin-2 (rIL-2). Fresh splenocytes cultured in media containing rIL-2 and cyclosporine A demonstrated a decrease in proliferation, cell cycle S-phase fraction, and cell yields compared to splenocytes cultured in media containing rIL-2 alone. Nourse et al. [23] observed that during T-cell proliferation, antigen-receptor and IL-2 signaling results in the increased expression of cyclins and activation of cyclinE/Cdk2 complexes. Kaplan et al. [24] using stat-6 (signal transducer and activator of transcription 6) deficient mice demonstrated that these proteins control IL-4-induced proliferation of activated T cells by regulating cell-cycle inhibitors. Modiano et al. [25] earlier demonstrated that IL-2 induced the expression and activity of Cdk4 and Cdk2 in human T cells allowing a progression through G1 and into S phase. Based on our and others' data, therefore, the cyclin inhibitor family of proteins may be important targets for altering the alloimmune response.

A number of CDK inhibitors have been discovered including p21WAF/CIP1, p15INK4B, and p27kip1. It is important to emphasize the fact that the effects of TGF-β on these CDK inhibitors have been established previously [26]. The most studied and significant cell cyclin inhibitor is p21WAF/CIP1, which is an effector protein that blocks CDKs and is induced by TGF-β [12,13]. Li et al. [27] studied the effect of TGF-β on different cell lines and observed that the induction of p21WAF/CIP1 by TGF-β was altered only in TGF-β sensitive cell lines. Though p21WAF/CIP1 appears to be one of the more important cell cyclin inhibitors, other cyclin inhibitors like p27 and p16 have been shown to inhibit cell cycle [28–30]. The specific target for p16 is the Cdk/4cyclin D complex [31]. In contrast to p21WAF/CIP1, the expression of p27 is not under transcriptional control and its mRNA expression remains unchanged during cell cycle [32]. Also, basal high levels of p27 but not p21WAF/CIP1 are observed in most quiescent cells and the inhibition of p27 levels precedes the progression through the cell cycle [32]. Though both p21WAF/CIP1 and p27 are critical in the response of cells to the mitogens, p21WAF/CIP1 provides a better balance between cyclins and cyclin inhibitors [33] stressing its significance in proliferation or immunosuppression. Furthermore Sugiyama et al. [34] observed that in rat hepatocytes TGF-β induced the expression of p21WAF/CIP1 but not of p27. The cells lacking p15 and p16 were sensitive to the growth inhibition by TGF-β whereas cells without p21WAF/CIP1 were resistant to the growth inhibition by TGF-β [35]. Genistein, an inhibitor of cell cycle progression, induced the expression of p21WAF/CIP1 but not of p27 in BALB/c 3T3 cells and B16-F1 melanoma cells [36]. Verlinden et al. [37] also observed that the treatment of MCF-7 cells with 1,25(OH) 2D3 resulted in the up-regulation of both p21WAF/CIP1 and p27 mRNA, however, treatment with anti-TGF-β neutralizing antibody did not alter the expression of p27 but completely abrogated p21WAF/CIP1 expression. These data indicated that the TGF-β signaling pathway was responsible for the up-regulation of p21WAF/CIP1 expression. The expression of p27 is induced by rapamycin in human T cells [21], however, later studies [38] demonstrated that p27-deficient T lymphocytes exhibited similar growth properties in response to mitogenic stimulation which was inhibited by both TGF-β and rapamycin, indicating that p27 might not be essential for these pathways.

We used A-549 cells, a human lung adenocarcinoma cell line, to study the induction of p21WAF/CIP1 by tacrolimus for several reasons. First, they are a well-established cell line used to study the biology of TGF-β [39]. Second, in our own work [3,4], we have shown that the tacrolimus inhibits the new DNA synthesis of this cell line and induces increased expression of TGF-β mRNA. Third, these cells are easy to transfect. Pretreatment of these cells with antisense oligonucleotide, directed against the transcription start site of TGF-β, abrogated CsA mediated effect on this cell line [40].

The current investigation describes a pathway novel to previously understood mechanisms for the action of tacrolimus. While p21WAF/CIP1 has been shown to be important in inhibiting cell cycle progression, we have not demonstrated that the induction of p21WAF/CIP1 is independent of TGF-β. Our recent studies [41], however, have established a role for p21WAF/CIP1 in modifying the immune response. Using p21WAF/CIP1 sense plasmids we could achieve direct over-expression of p21WAF/CIP1 and demonstrated that both in vitro and in vivo induction of p21WAF/CIP1 resulted in decreased lymphocyte proliferation and decreased expression of IL-2.

In summary, the data presented here directly link one of the primary immunosuppressive agents, tacrolimus, to a pathway that has heretofore not been a focus for

immunosuppressive strategies. These results provide a unifying mechanism of anti-proliferative/immunosuppressive effects of both tacrolimus and cyclosporine. It is likely that targeting the cyclins and/or cyclin inhibitors either pharmacologically or directly via gene therapy may achieve less toxic immunosuppression. Further studies on the direct in vivo effects of manipulating p21WAF/CIP1 in a transplantation model are in progress to establish a role for p21WAF/CIP1 in transplantation.

Acknowledgments

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