The effect of FK506 on transforming growth factor β signaling and apoptosis in chronic lymphocytic leukemia B cells

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ABSTRACT

Background

Loss of response to transforming growth factor-beta (TGF-β) is thought to contribute to the progression of chronic lymphocytic leukemia. Recent findings of over-activation of the TGF-β signal in FKBP12-knockout mouse prompted us to investigate whether FK506, the canonical ligand of FKBP, can activate the TGF-β signal in chronic lymphocytic leukemia.

Design and Methods

We studied 62 chronic lymphocytic leukemia samples from patients with Rai/Binet stage 0 to 4 disease. The TGF-β signal was investigated by western blotting and flow cytometry. The levels of Bcl2-family members and death-associated-protein kinase were also investigated by western blotting, whereas apoptosis was studied in flow cytometry. Down-modulation of FKBP12 was obtained by gene silencing with short interfering RNA.

Results

Twenty-two out of 62 chronic lymphocytic leukemia samples were sensitive to TGF-β-induced apoptosis. All but two of the responsive samples underwent apoptosis also when cultured with FK506, but not with cyclosporine. Thirteen samples that were not sensitive to TGF-β were sensitive to FK506. Overall, response to FK506 occurred in 33 samples. FK506 induced Smad2 phosphorylation and nuclear translocation. Accordingly, death-associated-protein kinase, a transcriptional target of Smad, was induced. At the same time, Bcl-2 and Bcl-xL levels decreased whereas the levels of Bim and Bmf increased. A loss of mitochondrial membrane potential preceded caspase activation and cell death. FK506 removed FKBP12 from its binding to the TGF-β-receptor. FKBP12 release activated the receptor-kinase activity as suggested by the enhanced levels of phospho-Smad found in cells depleted of FKBP12.

Conclusions

Our study shows that most chronic lymphocytic leukemia cells escape the homeostatic control of TGF-β and that FK506 restores the TGF-β signal in a proportion of non-responsive samples. We demonstrated that FK506 activates TGF-β receptor I kinase activity in chronic lymphocytic leukemia, which transduces apoptosis by a mitochondrial-dependent pathway.

Key words: FK506, chronic lymphocytic leukemia, TGF-β, apoptosis.

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Introduction

Transforming growth factor-beta (TGF-β) is a pleiotropic cytokine important in the control of cell growth and differentiation.[1-3] In normal cells, TGF-β acts as a tumor suppressor by inhibiting cell proliferation or promoting cellular differentiation or apoptosis.[2,4] Several lines of evidence support the view that the loss of sensitivity to TGF-β promotes leukemic transformation[5-7] and contributes to the clinical and biological progression of chronic lymphocytic leukemia (CLL).[8-10] This hematologic malignancy is a slowly progressing leukemia characterized by the gradual expansion of morphologically small, functionally inactive clonal B cells due to defective apoptosis.[11]

TGF -β signals to the nucleus by binding to a specific pair of membrane receptors, type I (TGFBR1) and type II (TGFBR2), which contain a cytoplasmic serine-threonine kinase domain.[2,12] Binding of the ligand to TGFBR2 results in the formation of a TGFBR1/TGFBR2 heteromeric complex and activation of TGFBR2 kinase.[2,12] Activation of TGFBR1 requires phosphorylation of the GS (glycine, serine) region by TGFBR2. Activated TGFBR1 specifically recognizes and phosphorylates signaling molecules that act downstream receptors (Smad) 2 and 3 or R-Smad.[2,12,13] In the basal state, R-Smad are retained in the cytoplasm. In the case of Smad2, this retention is mediated by interactions with the Smad anchor for receptor activation (Sara).[13] In addition to limiting Smad movements, contact with Sara occludes a region of Smad2 that mediates nuclear import.[13] Receptor-mediated phosphorylation not only increases the affinity of Smad2 for Smad4[12] but also decreases its affinity for Sara. Smad4 functions as a shared partner or Co-Smad and is required for the assembly of transcriptional complexes.[2,12] This process results in the release of Smad2 and unmasking of its nuclear import function thereby leading to rapid accumulation of the activated Smad complex in the nucleus.[2,12,13] Once in the nucleus, both R- and Co-Smad are able to activate transcription.[2,12-14]

TGF-β is apoptotic for hematopoietic cells.[15] Although the mechanism involved in TGF-β-induced apoptosis is not well known, mitochondria appear to be important mediators of this process.[16]

Identification of molecules able to restore the TGF-β response in B-CLL can have important implications in the treatment of this disease.[8-10] A network of regulatory inputs controls the TGF-β signaling pathway.[2] A recent study of fibroblasts from FK506 binding protein (FKBP) 12-knockout mice showed that the TGF-β pathway is overactive in cells lacking this protein.[17] FKBP12 is a common inhibitor of the TGF-β family type I receptors,[18-20] it binds to part of the GS region towards the N-terminal end of the serine-threonine kinase domain of *TGFBR1*[2,12] thereby blocking access to activators. Ligand binding induces the release of FKBP12, which is essential for propagating the signal.[2,12,17,18,20] The finding that FKBP12-binding molecules, such as FK506 and rapamycin, are able to promote receptor transphosphorylation,[18,20] prompted us to investigate whether FK506, the canonical ligand of FKBP12,[19,21] could restore TGF-β response and stimulate apoptosis of CLL cells.

Design and Methods

Cell culture and reagents

CLL cells were isolated from the heparinized blood taken from 62 out-patients, after informed consent, by differential centrifugation through a Ficoll-Hypaque density gradient (ICN Flow, Opera, Italy). The study was approved by the Ethics Committee of Federico II University of Naples. Only CLL patients who had >80% CD19 cells co-expressing CD5 were included in the study. Cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS; ICN Flow). FK506, cyclosporine and TGF-β (Sigma Aldrich, St. Louis, MS, USA) were used at the doses indicated in the Results section. The peptide caspase 3 inhibitor Z-Asp-Glu-Val-Asp fluoromethyl ketone (Z-DEVD-fmk, Sigma Aldrich) was used at the dose of 20 μM. Table 1 shows the clinical profiles of the patients studied, related to the time of blood sampling. The patients did not receive any therapy in the 6 months, at least, preceding blood collection. Peripheral blood lymphocytes were isolated from the heparinized blood of healthy donors by differential centrifugation through a Ficoll-Hypaque density gradient; B lymphocytes were sorted from peripheral blood lymphocytes with a BD FACSAria™ (BD Biosciences, San Jose, CA USA). The purified population was ≥ 97% $CD20^+$.

Cell lysates and western blot assay

Whole cell lysates were prepared by homogenization in modified RIPA buffer [150 mM sodium chloride, 50 mM Tris-HCl, pH 7.4, 1 mM ethylenediamine tetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluorite (PMSF), 1% Triton X-100, 1% sodium deoxycholic acid, 0.1% sodium dodecylsulfate (SDS), 5 μg/mL aprotinin and 5 μg/mL leupeptin]. Cell debris was removed by centrifugation. Cell fractionation was obtained as described elsewere[22] with small changes. Briefly, cells were washed twice with phosphate-buffered saline (PBS) and resuspended in 200 μL buffer A [10 mM TRIS HCl pH 7.4, 10% glycerol, 1mM $MgCl_2$, 1 mM PMSF, 5 μg/mL aprotinin and 5 μg/mL leupeptin] for 15 min on ice, before adding 2 μL of 10% Nonidet P-40. The cells were vortexed for 20–30 sec and spun for 10 min at 3000 rpm to spin down the nuclei. The cytoplasmic fraction was saved, and the nuclear pellet was washed once with buffer A. Nuclei were resuspended in modified RIPA buffer and extracted at 4°C for 30 min. Cell lysates were run in 10% SDS in polyacrylamide gel electrophoresis (PAGE) along with a molecular weight marker and transferred onto a membrane filter (Cellulosenitrate, Schleider and Schuell, Keene, NH, USA), which was incubated with the primary antibody.

The rabbit polyclonal antibodies against phospho-Smad2 (Ser 465/467) (Chemicon Temecula, CA, USA), caspase3 (Pharmigen/Becton Dickinson, San Diego, CA, USA), Smad 2,3 (H-465) and Bim (H-191) (Santa

Cruz Biotechnology, Santa Cruz, CA, USA); the mouse monoclonal anti-Bcl-xL (H-5), Bcl-2 (100), Smad4 (B-8), histone H1 (AE-4) (Santa Cruz Biotechnology), death-associated potein kinase (DAPK-55) (Sigma Aldrich), EF-1· (Upstate, Charlottesville, Virginia, USA) and the goat polyclonal anti-Bmf (Santa Cruz Biotechnology), were used diluted 1:500-1:1000. The blots were developed with an electrochemiluminescence system (Supersignal West Pico, Celbio, PIERCE, Rockford, IL, USA).

Immunofluorescence

For nuclear immunofluorescence, nuclei were purified from CLL cells by hypotonic lysis of plasma membrane and sucrose gradient. Briefly, cells were washed twice with PBS and resuspended in 200 μL buffer A [10 mM TRIS HCl pH 7.4, 10% glycerol, 1 mM $MgCl_2$, 1 mM PMSF, 5 μg/mL aprotinin and 5 μg/mL leupeptin] for 15 min on ice, before 2 μL of 10% Nonidet P-40 were added. The cells were vortexed for 20-30 sec and spun for 10 min at 3000 rpm to spin down the nuclei. The pellet was washed once with buffer A, resuspended in buffer A and a cushion carefully laid beneath it (30% sucrose w/v in buffer A). After centrifugation at 6000 rpm for 15 min (4° C), the supernatant was removed and the final pellet washed with buffer B [20 mM TRIS HCL pH 8.0, 75 mM NaCl, 0.5 mM EDTA pH 8.0, 0.85 mM dithiothreitol (DTT), 0.125 mM PMSF]. Nuclei isolated from $10\text{-}20\times10^6$ BCLL cells were subjected to immunostaining for 30 min at 4°C and analyzed by a FACScan 30 (BD) flow cytometer.

For intracellular staining with anti-phosphoSmad 2 antibody, B cells were fixed with 2% paraformaldehyde in Tris buffered saline (TBS) (10× TBS = 0.5M Tris Base, 9% NaCl, pH 7.6) for 20 min and permeabilized with 0.2%TRITON ×100 in TBS for 3 min in ice. Afterwards, cells were incubated with anti-phosphoSmad2 for 30 min at 4°C. After the cells had been washed, phosphoSmad2 was detected by immunostaining with a secondary fluorescein isothiocyanate (FITC) -conjugated anti-rabbit antibody and measured in flow cytometry.

Immunoprecipitation of membranes

Cells were osmotically lysed in distilled water and subjected to three cycles of rapid freezing and thawing. During thawning, extract was sonicated for 10 min. After obtaining a homogeneous suspension, protein concentration was determined using the Bradford method and 500 μg of protein extract were precleared for 1 hour. For immunoprecipitation, 15 μg anti-TGFBR1 (rabbit polyclonal H-100) or anti-FKBP12 (goat polyclonal N-19), (Santa Cruz Biotechnology) were added together with 25 μL protein A-Sepharose (Santa Cruz Biotechnology) and precipitation took place overnight with rotation at 4°C. Samples were separated by 14% SDS-PAGE along with a molecular weight marker and transferred onto a membrane filter.

Cell transfection and short interfering (si)RNA

Twenty-four hours before transfection of siRNA corresponding to the target sequence GCGGCTAGGTGTTATCTGA of the FKBP12 gene (Qiagen, CA, USA) or of a scrambled duplex as a control, cells were incubated in medium without antibiotics at the concentration of 5×10^5/mL. The siRNA or the scrambled oligo was transfected at the final concentration of 50 nM using

Table 1. Patients' profiles and response of samples.

	BINET/RAI	LDT[a]	Bulky disease	R TGF-β[b]	R FK506[c]
1.	0/A	Low	-	-	Yes
2.	0/A	Low	-	-	Yes
3.	0/A	Low	-	-	Yes
4.	0/A	Low	-	-	Yes
5.	0/A	Low	-	-	-
6.	I/A	Low	-	-	Yes
7.	0/A	Low	-	-	-
8.	0/A	Low	-	-	-
9.	0/A	Low	-	-	-
10.	0/A	Low	-	Yes	Yes
11.	0/A	Low	-	Yes	Yes
12.	0/A	Low	-	Yes	Yes
13.	0/A	Low	-	Yes	Yes
14.	0/A	Low	-	Yes	Yes
15.	0/A	Low	-	Yes	Yes
16.	I/A	Low	-	Yes	Yes
17.	I/A	Low	-	Yes	Yes
18.	I/A	Low	-	Yes	Yes
19.	I/A	Low	-	-	Yes
20.	I/A	Low	-	-	-
21.	I/A	Low	-	-	-
22.	I/A	Low	-	-	-
23.	I/A	Low	-	-	-
24.	I/A	Low	-	-	-
25.	I/B	Low	-	-	Yes
26.	I/B	Low	-	-	-
27.	I/B	Low	-	-	-
28.	I/B	Low	-	-	-
29.	I/B	Low	-	-	-
30.	I/B	Low	-	Yes	Yes
31.	I/B	Low	-	Yes	Yes
32.	II/A	Low	-	Yes	Yes
33.	II/B	Low	-	Yes	-
34.	II/B	Low	-	Yes	Yes
35.	II/B	Low	-	Yes	Yes
36.	II/B	n/a	-	Yes	Yes
37.	II/B	Low	-	Yes	Yes
38.	II/B	Low	-	Yes	Yes
39.	II/B	Low	-	-	Yes
40.	II/B	High	-	-	Yes
41.	II/B	Low	-	-	Yes
42.	II/B	Low	-	-	-
43.	II/B	Low	-	-	Yes
44.	II/B	High	-	-	Yes
45.	II/B	Low	-	-	-
46.	II/B	Low	-	-	-
47.	II/B	Low	-	-	-
48.	II/B	Low	-	-	-
49.	II/C	Low	-	Yes	-
50.	III/C	n/a	-	-	-
51.	III/C	Low	-	-	-
52.	III/C	High	Yes	-	-
53.	III/C	Low	-	Yes	Yes
54.	III/C	Low	-	Yes	Yes
55.	IV/C	Low	-	Yes	Yes
56.	IV/C	n/a	-	-	-
57.	IV/C	Low	-	-	-
58.	IV/C	Low	-	-	-
59.	IV/C	High	Yes	-	-
60.	IV/C	High	Yes	-	Yes
61.	IV/C	High	Yes	-	-
62.	IV/C	High	Yes	-	-

[a]LDT: lymphocyte doubling time (Low: >12 months); [b]R TGF-β: in vitro response to TGF-β; [c]R FK506: in vitro response to FK506.

Metafectene (Biontex, Munich, Germany) according to the manufacturer's recommendations and after 48 days, cells were harvested to prepare cell lysates. The effect of siRNA on protein expression was confirmed by western blotting.

Analysis of apoptosis

Phosphatidylserine externalization was investigated by annexin V staining. Briefly, 1×10^5 cells were resuspended in 100 μL of binding buffer (10 mM Hepes/NaOH pH 7.5, 140 mM NaCl, 2.5 mM $CaCl_2$) containing 5 μL of annexin V-FITC (Pharmigen/Becton Dickinson, San Diego, CA, USA) for 15 min at room temperature in the dark. Then 400 μL of the same buffer were added to each sample and the cells were analyzed with a Becton Dickinson FACScan flow-cytometer. The lipophilic cation 5,5,6,6 tetrachloro-1,1,3,3 -tetraethylbenzimidazol-carbocyanine iodide (JC-1) was utilized to study mitochondrial membrane potential. In this procedure, the color of the dye changes from orange to green as the membrane potential decreases, due to JC-1 aggregates dissolving in monomers. Briefly, 5×10^5 cells were incubated for 10 minutes at 37°C with 10 μg/mL JC-1 (Molecular Probes, Leiden, The Netherlands), washed, and analyzed by flow cytometry.

Statistical analysis

The results of continuous variables are reported as medians and interquartile ranges. Frequencies are used for categorical data. The statistical significance of differences between groups of continuous data was estimated using the Mann-Whitney non-parametric unpaired test. Fisher's exact test was used to assess differences between categorical variables. p values ≤0.05 were considered statistically significant. The statistical analysis was performed using SPSS statistical package (SPSS Inc. Chicago, IL, USA).

Results

FK506 induces death of B-CLL cells

To investigate the effect of FK506 on CLL cell apoptosis, we cultured the cells with TGF-β (10 ng/mL), FK506 (100 ng/mL) or the cyclophilin A inhibitor cyclosporine (300 ng/mL) and measured cell death by annexin V staining and flow cytometry, after 24 h of incubation. As expected, we found spontaneous apoptosis of CLL cells after their *in vitro* culture.[23] TGF-β increased basal apoptosis by at least 20% in 22 of 62 samples. Figure 1A is a graphic representation of median values and interquartile ranges of apoptosis in samples that were and were not responsive to TGF-β. The median apoptosis in non-responsive samples was 22.0% (range 16.0-27.7%) and 22.0% (range 17.2-26.0%) in the absence and presence of TGF-β, respectively. The median apoptosis in responsive samples was 26.0% (range 20.7-29.2%) and 35.5% (range 30.7-43.7%) in the absence or presence of TGF-β (p<0.001), respectively.

Table 1 shows the CLL patients' profiles in relation to the response of the samples. Interestingly, the proportion of patients with low (>12 months) lymphocyte doubling time (LDT) was statistically higher (p=0.04) in TGF-β responders (100% low LDT) than in non-responders (81% low LDT). LDT is defined as the time needed for lymphocytes to double in number from the amount present at diagnosis.[24] This finding supports the concept that TGF-β inhibits cell growth. Regarding response to FK506, basal apoptosis was increased in 33 samples (Figure 1B). All but two of the samples sensitive to TGF-β were also sensitive to FK506. Median apoptosis in FK506-non-responsive samples was 22.0% (range 17.5-27.5%) and 22.0% (range 16.5-28.0%) in the absence or presence of the macrolide, respectively. The median apoptosis in FK506-responsive samples was 26.0% (range 21.5-30.0%) and 39.5% (range 29.7-42.2%) in the absence or presence of the drug (p<0.001), respectively. Interestingly, FK506 and TGF-β induced a similar degree of apoptosis in responsive samples. Cyclosporine did not induce cell death suggesting that the mechanism responsible for immunosuppression, i.e. calcineurin inhibition, was not involved in activation of the apoptotic machinery. FK506-induced apoptosis was remarkable at doses ≥10 ng/mL (Figure 1C) and reached the maximum level as early as 16 h after incubation (Figure 1D).

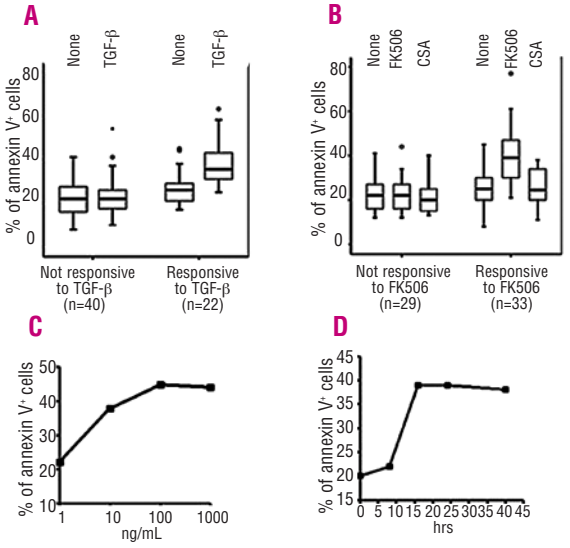

Figure 1. FK506 induces apoptosis of cells from patients with CLL. Graphic representation of median values and interquartile ranges of apoptosis measured by annexin V staining and flow cytometry. Cells were cultured with or without TGF-β (10 ng/mL). (A) With and without FK506 (100 ng/mL) or cyclosporine (300 ng/mL). (B) After 24 h cells were harvested, incubated with annexin V-FITC and analyzed in a flow cytometer. Each experiment was performed in triplicate. (C) Dose/response curve of FK506-induced apoptosis in a responsive sample. Cells were cultured with FK506 at the indicated doses. After 24 h of incubation, cells were harvested and apoptosis was determined by annexin V staining and flow cytometry. The experiment was performed in triplicate; the graphic shows the mean values of the triplicate experiments. (D) Kinetics of FK506-induced apoptosis. Cells were cultured with 100 ng/mL FK506 and were harvested at different times (8, 16, 24 and 40 h) and analyzed for apoptosis with annexin V staining and flow cytometry. The experiment was performed in triplicate and the mean results are reported.

FK506 activates the Smad complex

To test our hypothesis that the apoptotic response to FK506 of BCLL cells resulted from activation of the TGF-β signal, we investigated activation of Smad proteins by measuring the levels of phospho-Smad2 in cells incubated with FK506. Figure 2 shows western blot assays of three different samples, the first of which responded to both FK506 and TGF-β, the second one to FK506 but not to TGF-β and the third one did not respond to FK506. Phospho-Smad2 levels were increased in samples undergoing apoptosis, suggesting that activation of the TGF-β signaling pathway promoted cell death in FK506 cultures. To determine whether this response to TFG-β by CLL cells corresponded to a physiological effect, we studied the response of normal peripheral B lymphocytes to the cytokine. As shown in Figure 3A, phospho-Smad2 levels were increased after 1 h of incubation of purified B lymphocytes with TGF-β. Similarly, FK506 activated Smad in the same cells. Apoptosis analysis showed a slight increase of cell death in both TGF-β- and FK506-cultures.

The levels of phospho-Smad2 in CLL cells cultured with FK506 increased as early as 10 min after incubation, peaked at 3 h and decreased after 4 h (Figure 4A). Nuclear translocation of Smad-complex was found in isolated nuclei stained with anti Smad4 and analyzed by flow cytometry (Figure 4B). Smad nuclear translocation was confirmed by western blot assay of lysates obtained by cell fractionation. As shown in Figure 4C, the increase of Smad4 in nuclei was accompanied by its decrease in the cytoplasm. We used anti-histone H1 as a nuclear loading control and monoclonal antibody against the protein synthesis elongation factor, EF-1α,[25] as a cytosolic loading control.

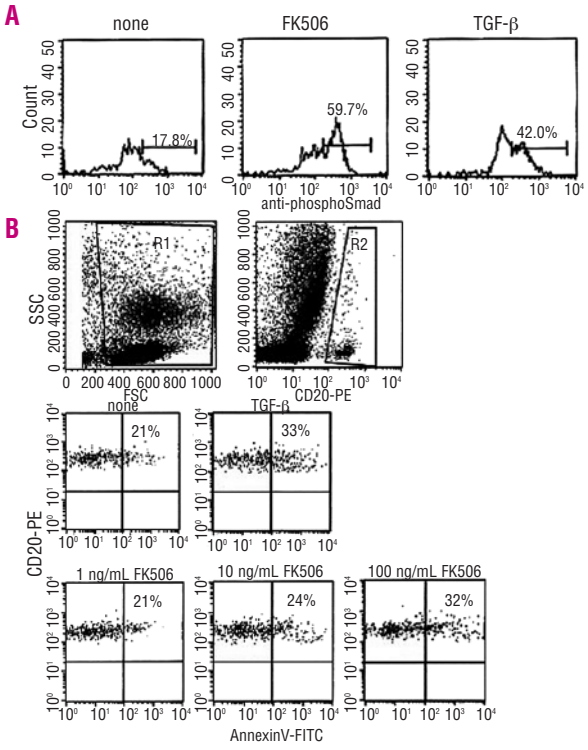

Figure 3. Effect of TGF-β and FK506 on normal B lymphocytes. (A) Flow cytometric analysis of phospho-Smad levels in purified B lymphocytes ($CD20^+$ ≥97%) cultured in the absence or presence of 10 ng/mL TGF-β or 100 ng/mL FK506 for 1 h. (B) Flow cytometric analysis of apoptosis of B lymphocytes cultured with 10 ng/mL TGF-β and FK506 at different doses. Total peripheral blood lymphocytes were incubated with the indicated doses of the reagents for 48 h. Then, analysis of apoptosis was performed in double staining with annexin V-FITC and CD20-PE. Lymphocytes were identified using a FSC/SSC dual parameter dot plot (gate R1). All events in R1 were sent to a second display of CD20/SSC in which $CD20^+$ cells (gate R2) were easily distinguished from non-B cells. Annexin V expression was measured using a logical gate (R1 and R2) which allows only the events which are in both R1 and R2 to be analyzed. The data presented are representative of three independent experiments.

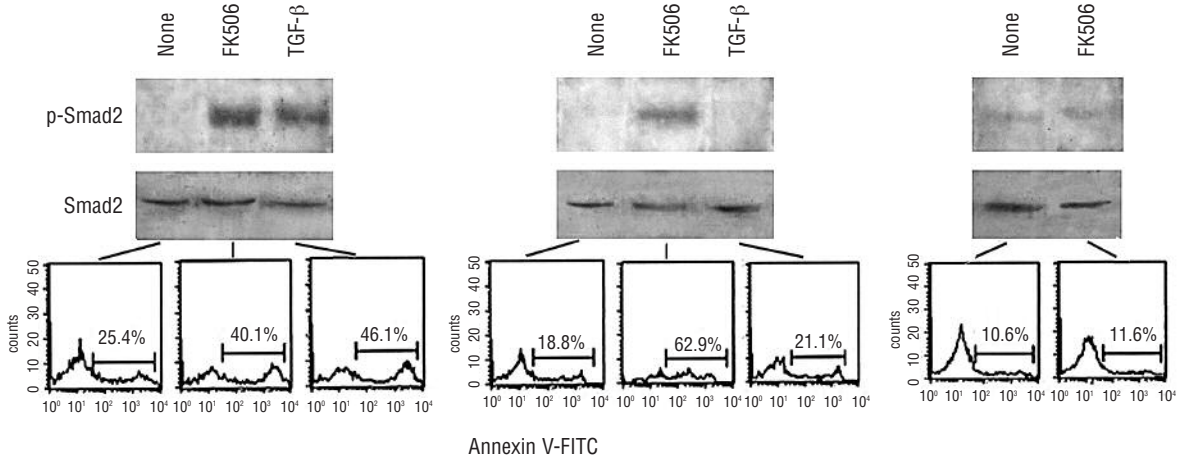

Figure 2. FK506-induced apoptosis is preceded by Smad2-phosphorylation. Western blot assay of phospho-Smad2 at Ser 465-467 in whole cell lysates (30 μg) from three different CLL samples. The samples used were, in order, n. 30 (TGF-β responsive) and n. 60 and 45 (TGF-β non-responsive). Five other samples analyzed did respond to FK506 and showed phosphorylation of Smad2. Cells were cultured with the indicated reagents (100 ng/mL FK506, 10 ng/mL TGF-β) and, after 3 h, a portion of cells was harvested for whole cell lysate preparation, whereas the remaining cells were harvested after a further 21 h and analyzed for apoptosis with annexin V staining and flow cytometry.

FK506 modulates the expression levels of both pro- and anti-apoptotic members of the Bcl-2 family of proteins

Death-associated protein kinase is a transcriptional target of Smad[16] that links Smad to TGF-β mitochondrial events.[16] We used western blot assays to investigate whether FK506 increased the levels of this protein in CLL cells. We also evaluated the expression levels of the anti-apoptotic Bcl-2 and Bcl-xL, and the pro-apoptotic BH3-only molecules, Bim and Bmf, which are implicated in commitment to TGF-β-induced apoptosis.[26-28] We found that the appearance of death-associated protein kinase was accompanied by a decrease of Bcl-2 and Bcl-xL and an increase of Bim (Figure 5A). Bim is an essential regulator of lymphoid system homeostasis and appears to be essential for induction of B-cell apoptosis. All the three Bim isoforms, Bim_{EL}, Bim_{L} and Bim_{S}, which exert comparable activity,[27,28] were upregulated by FK506 in two different samples (Figure 5B). Similarly, the levels of expression of Bcl-2 modifying factor or Bmf were also increased (Figure 5C).

FK506 induces mitochondrial depolarization and activation of caspase 3

In accordance with the modulation of Bcl-2 family member proteins, the study of mitochondrial membrane potential with the lipophilic cation JC-1 showed depolarization in FK506 cultures, whereas this did not occur when cyclosporine was added to the culture medium (Figure 6A). A dose-response effect was observed by stimulating the cells with different FK506 doses (Figure 6B). Figure 6C shows the kinetics of FK506-induced mitochondrial depolarization in a responsive sample. More than 70% of cells depolarized after 8 h. Activation of caspase3 is a hallmark of apoptosis and represents the converging point of both intrinsic (downstream mitochondria) and extrinsic (downstream death receptors) pathways. Previous reports implicate caspase3 in TGF-β-induced apoptosis;[29] we, therefore, investigated whether the active form of caspase3 appeared in cells incubated with FK506. Western blot assay of whole cell lysates prepared after 16 h of incubation revealed the presence of activated caspase3, which resulted from cleavage adjacent to Asp175 (Figure 7).

FK506 activates TGFBR1 by removing FKBP12 from TGFBR1

Displacement of FKBP12 from its binding to the GS region of TGFBR1 is essential for activation of the kinase activity.[20] By co-immunoprecipitation studies, we found that FK506 interfered with the FKBP12/TGFBR1 interac-

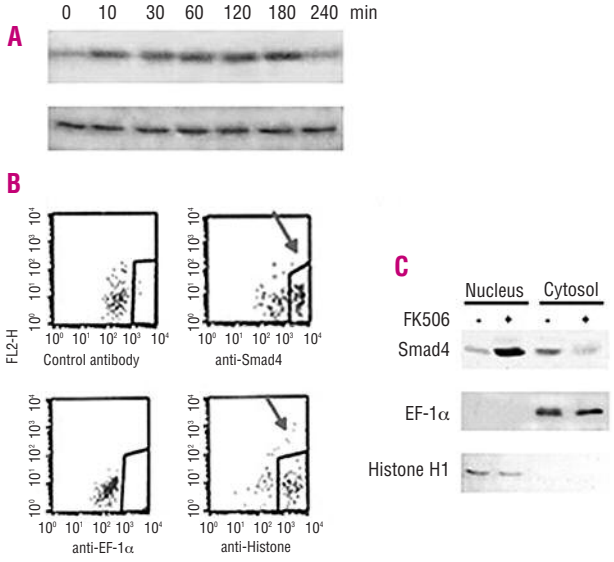

Figure 4. FK506 activates nuclear translocation of Smad complex. Kinetics of FK506-induced Smad2 phosphorylation. (A) Western blot assay of phospho-Smad2 (Ser 465-467) levels in whole cell lysates (30 μg) of CLL cells (sample #30,TGF-β responsive) incubated with 100 ng/mL FK506 for the indicated times. (B) Flow cytometric histograms of Smad4 expression in purified CLL nuclei. Cells (sample #30,TGF-β responsive) were incubated with 100 ng/mL FK506. After 3 h, cells were harvested and purified nuclei were subjected to indirect immunofluorescence with anti-Smad4. Nuclear autofluorescence was localized between 10^2–10^3 decades of the logarithmic scale, whereas Smad4-positive nuclei appeared in the 10^4 decade. Staining with anti-histone or anti EF-1α served as a control of nuclear purification. (C) Western blot assay of Smad4 levels in cell lysates (20 μg) obtained by CLL cell fractionation (sample #60, not responsive to TGF-β) after 3 h incubation with 100 ng/mL FK506. Anti-histone H1 or anti EF-1α served as a loading control for nucleus and cytosol, respectively.

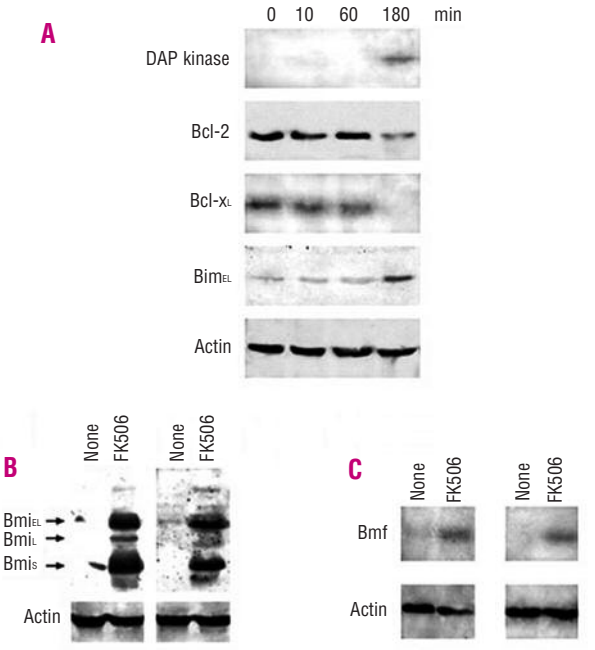

Figure 5. FK506 induces death-associated protein (DAP) kinase and modulates several members of the Bcl-2 protein family. (A) Western blot assay of DAP kinase, Bcl-2, Bcl-XL and Bim levels in whole cell lysates (30 μg) of CLL cells (sample #30, TGF-β responsive) incubated with 100 ng/mL FK506 for 3 h. (B) Western blot assays of the three Bim isoform levels in whole cell lysates (30 μg) of CLL cells from two samples (#30, responsive to TGF-β and #39, not responsive to TGF-β) incubated with 100 ng/mL FK506 for 10 h. (C) Western blot assay of Bmf levels in whole cell lysates (30 μg) of CLL cells from two different samples (#30, responsive to TGF-β and #39, not responsive to TGF-β) incubated with 100 ng/mL FK506 for 10 h.

tion. As shown in Figure 8A, TGFBR1 co-immunoprecipitated with FKBP12 in unstimulated- but not in FK506-stimulated peripheral blood lymphocytes, suggesting that the drug removed FKBP12. In order to investigate whether this effect was sufficient to activate the kinase activity, we depleted the cells of FKBP12 and measured the levels of phosphorylated Smad2. Figure 8B shows that a remarkable increase in phosphoSmad levels was associated with reduced levels of FKBP12. Consistent with the activation of the TGF-β signal, enhanced levels of Bim_{EL} were also found. The effect of FKBP12 down-modulation on activation of the TGF-β signal was also confirmed in CLL. Figure 9 shows results from a sample responsive to FK506. Panel A shows flow cytometric histograms of annexin V staining of CLL cells after culture for 24 h in the absence or presence of 100 ng/mL FK506 and with or without the caspase3 inhibitor Z-DEVD-fmk. The percentage of FK506-induced cell death appeared remarkably reduced by the caspase inhibitor. In the experiments whose results are shown in panel B, the same cells were transfected with FKBP12 siRNA or a scrambled duplex as a control. After 48 h, total lysates were prepared and analyzed in western blot assays to measure the levels of FKBP12 and pSmad. Reduced levels of FKBP12 were accompanied by enhanced levels of pSmad. After a further 24 h, apoptosis was measured by annexin V staining (panel C). FKBP12 down-modulation produced levels of apoptosis comparable to those induced by FK506. Similarly to FK506 cultures, Z-DEVD-fmk decreased apoptosis of cells transfected with FKBP12 siRNA. These results were confirmed in two other independent experiments.

Discussion

There is growing interest in understanding and therapeutically targeting TGF-β-mediated processes in cancer[1,3-5] including hematologic malignancies.[1] Herein, we show that FK506 activates TGF-β-signaling in CLL. FK506, or fujimycin, the canonical ligand of FKBP12,[19] is a 23-membered macrolide lactone mainly used to prevent organ rejection after allogeneic transplants.[21] B lymphocytes from subjects with CLL show heterogenous responses to TGF-β;[10] accordingly, our results indicated that 35.4% of the CLL samples analyzed (22/62) were sensitive to the TGF-β apoptotic effect. FK506 restored response to TGF-β in a further 17.7% of samples, thereby increasing the percentage of responses to 53.2%. Our data show that normal B lymphocytes also responded to TGF-β with low levels of apoptosis, which is in accordance with the notion that TGF-β is an important regulator of hematopoietic homeostasis. The response to TGF-β, found in some of the CLL samples, appeared, therefore, to be a conserved physiological effect.

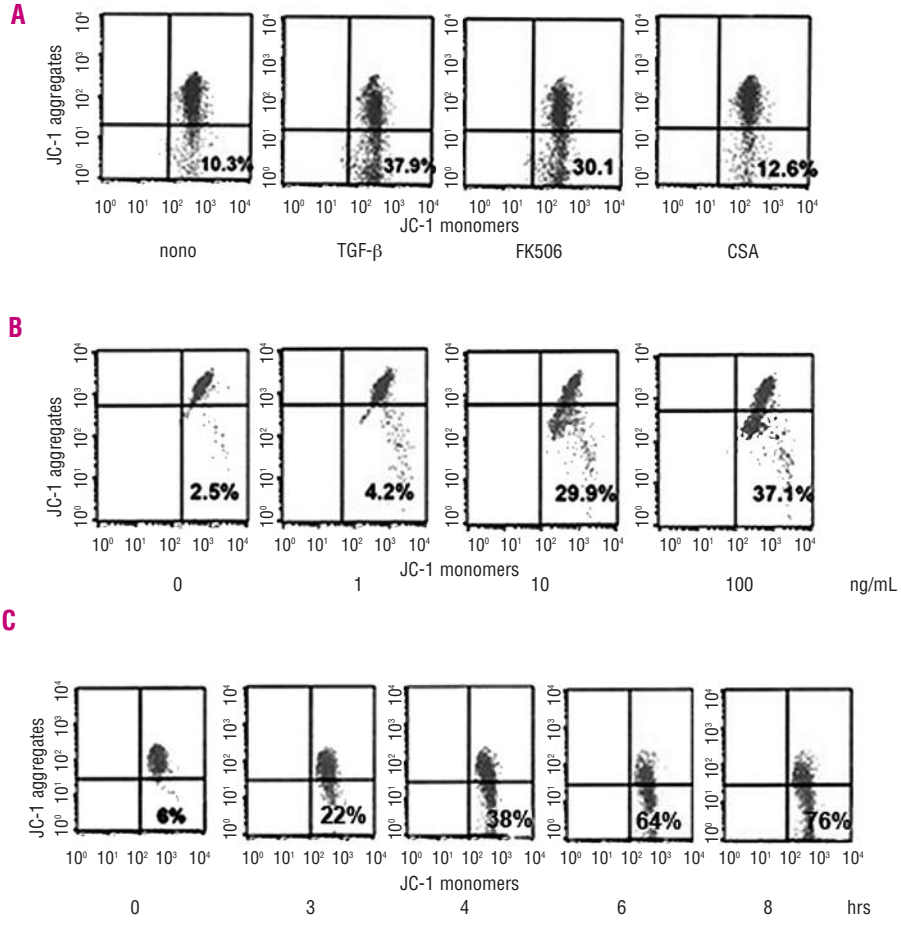

Figure 6. FK506 provokes mitochondrial depolarization. Analysis of mitochondrial membrane potential in three chronic lymphocytic leukemia samples with the lipophilic cation JC-1. The samples used were, in order, #10, #17 and #12 (TGF-β responsive). The percentage of depolarized cells was determined by calculating the amount of JC-1 monomers in flow cytometry. (A) Cells were incubated with or without 10 ng/mL TGF-β, 100 ng/mL FK506, and 300 ng/mL cyclosporine for 8 h. (B) Cells were incubated with the indicated doses of FK506 for 8 h. (C) Cells were incubated with 100 ng/mL FK506 for the indicated times.

FK506 activates TGF-β receptor kinase activity.[18,20] In CLL cells cultured with FK506, the appearance of phosphorylated Smad2 was detected as early as 10 min after incubation. The level of phosphorylated Smad2 peaked after 3 h and disappeared after 4 h. Smad complexes were found in the nucleus after 3 h of incubation with FK506. At the same time, there was an increase in levels of death-associated protein kinase, which is a target of transcriptional activation by Smad.[16] In accordance with reports that Bim is activated and functions as an important initiator of TGF-β-induced apoptosis in both a human gastric carcinoma cell line[26] and a B-cell line,[28] Bim was upregulated in CLL cells cultured with FK506. Bmf is another BH3-only protein implicated in TGF-β-induced apoptosis[27] which plays a role in regulating the growth and survival of B cells[28] and CLL cells.[29] Bim and Bmf are activators of Bax-Bak,[28] which are pro-apoptotic Bcl-2 members that are the downstream effectors controlling the mitochondrion-dependent cell death program. It is now well established that the role of anti-apoptotic Bcl-2 members correlates with their ability to sequester BH3-activators, thereby preventing the acti-

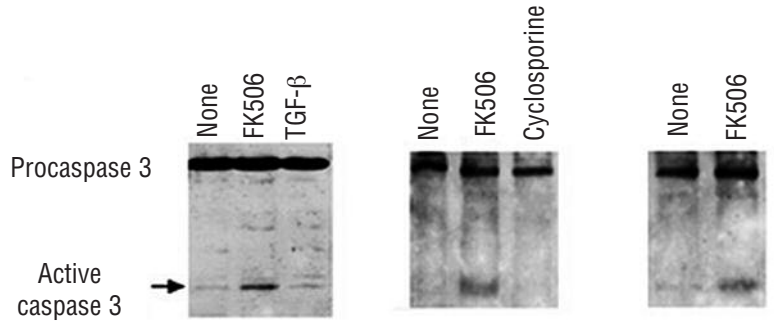

Figure 7. FK506 activates caspase 3. Western blot assay of active caspase 3 in whole cell lysates (30 μg) obtained from chronic lymphocytic leukemia cells of three different samples incubated with the indicated reagents (100 ng/mL FK506, 10 ng/mL TGF-β, 300 ng/mL cyclosporine) for 16 h. The samples used were, in order, #60, #3, and #43 (not responsive to TGF-β). The primary antibody used in this assay recognizes procaspase 3 and the activated caspase3 resulting from cleavage adjacent to Asp175.

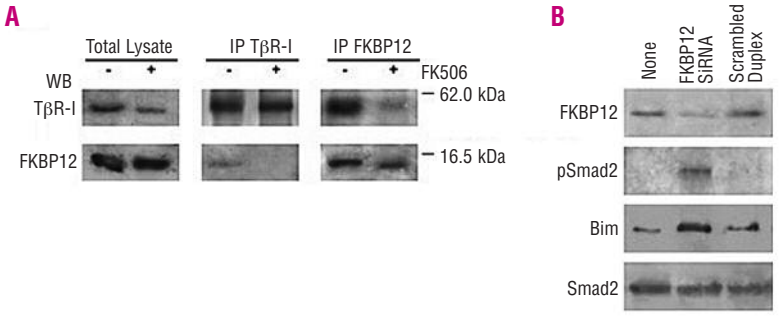

Figure 8. FK506 activates TβR-I by removing FKBP12. (A) FKBP12/TβR-I interaction in peripheral blood lymphocytes. Cell lysates were subjected to immunoprecipitation (IP) with anti-TβR-I or FKBP12 antibody. Immunoprecipitated and total lysates were then subjected to western blotting with anti-TβR-I or -FKBP12. (B) Western blot assay of FKBP12, phosphoSmad2 and Bim levels in total lysates prepared from non-transfected peripheral blood lymphocytes and from peripheral blood lymphocytes transfected with FKBP12 siRNA or a scrambled duplex as a control. Smad2 was used as the loading control.

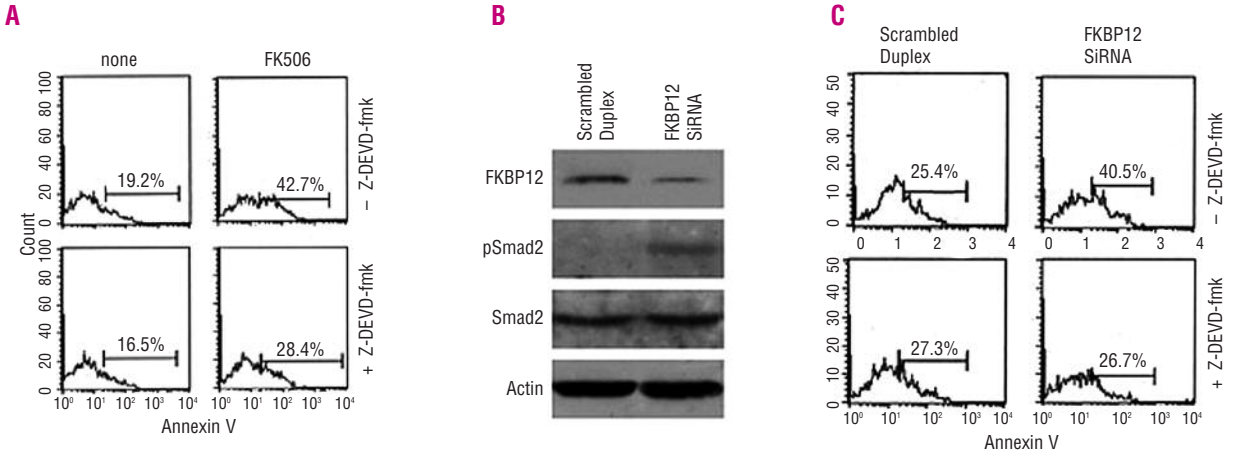

Figure 9. FKBP12 down-modulation causes apoptosis of CLL cells. (A) Flow cytometric histograms of annexin V staining. Chronic lymphocytic leukemia cells were cultured in the absence or the presence of 100 ng/mL FK506 and with or without the caspase3 inhibitor Z-DEVD-fmk (20 μM), then apoptosis was measured after 24 h. (B) The same cells were transfected with FKBP12 siRNA or a scrambled duplex as a control. After 48 h, total lysates were prepared and analyzed by western blotting to measure the levels of FKBP12 and pSmad. (C) Flow cytometric histograms of annexin V staining of chronic lymphocytic leukemia cells transfected with FKBP12 siRNA or a scrambled duplex as a control. Forty-eight hours after transfection, 20μM Z-DEVD-fmk was added to the cultures and, after a further 24 h, apoptosis was measured.

vators from triggering the lethal action of Bax-Bak.[30] FK506-induced apoptosis was preceded by a rapid decrease of Bcl-2 and Bcl-xL. In such condition, it is feasible that Bim caused loss of mitochondrial membrane potential and apoptosis in CLL because of lack of the neutralizing action of Bcl-2 and BclxL.

FK506 removed FKBP12 from the cytoplasmic tail of the TGF-β receptor. This displacement was apparently sufficient to activate the receptor kinase activity, as suggested by the appearance of posphoSmad in cells depleted of FKBP12. An inhibitory effect of TGFBR1 function was observed in FKBP12-overexpressing cells by Chen and colleagues.[18] The increased expression of FKBP12, that we found in some cases (*data not shown*) may, therefore, be one of the reasons for a lack of or no response to TGF-β. Reduced expression of TGFBR1 has been found by several authors in CLL[8-10] and suggested as a cause of insensitivity to TGF-β. It is feasible that in a condition of low receptor level, even normal levels of FKBP12 may be inhibitory and, possibly, the signal might be enhanced by FK506 also in these cases. Moreover, receptor mutations that affect binding but not the kinase domain might also account for response to FK506 but not TGF-β. The absence of response to both TGF-β and FK506 is in accordance with the findings of recurrent mutations in the signal sequence of TGFBR1, which are responsible for defective kinase activity.[31]

Taken together, these findings suggest that FK506 induced activation of the TGF-β signal transduction pathway. In accordance with several reports suggesting that loss of response to TGF-β might provide a selective advantage to CLL B lymphocytes and contribute to the expansion of neoplastic clone,[8-10] our study showed an association between response to TGF-β and a LDT greater than 12 months, suggesting that the cytokine inhibited tumor cell growth. Therefore, rescue of TGF-β response in CLL by FK506 could represent a breakthrough in the treatment of this common hematologic malignancy.

Previous studies have shown that FK506 can reverse the multidrug resistance phenotype[32] and does not suppress bone marrow activity, whereas it apparently stimulates very early hematopoietic progenitor cells.[33] Consequently, FK506 appears to be a promising agent that deserves future investigation in combined chemotherapy.

Immunosuppressive agents have recently been used in anti-cancer therapy with rapamycin and its analogs.[34] However, our study indicates that the apoptotic effect of FK506 occurs irrespectively of an immunosuppressive mechanism. Consequently, it is feasible that derivatives of FK506 that have the same FKBP12-binding properties as FK506 but lack the calcineurin binding domain, and thus lack functional immunosuppressant activity, could exert the same effect as FK506 in CLL.

In conclusion, our study shows that most CLL cells escape the homeostatic control of TGF-β. This finding is common to many tumors[3-5] in which mutations of components of the TGF-β response pathway hamper restoration of the signal. We demonstrate that FK506 can re-activate the TGF-β signal in CLL, thereby increasing the proportion of responsiveness.

Authorship and Disclosures

SR, MM, RB, AD'A, GC performed the experimental work, acquired, analyzed and interpreted data and critically revised the article giving their final approval; FC provided samples and informed consent, performed the experimental work, acquired, analyzed and interpreted data and critically revised the article giving his final approval; RL provided analytical tools and statistical analysis, performed the experimental work, acquired, analyzed and interpreted data and critically revised the article giving his final approval; MFR designed and performed the experimental work, analyzed and interpreted data and wrote the article, revising it critically for important intellectual content. The authors reported no potential conflicts of interest.

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