Regulation of tumor necrosis factor cytotoxicity by calcineurin

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Abstract Cyclosporin (CsA) inhibits mitochondrial death signaling and opposes tumor necrosis factor (TNF)-induced apoptosis in vitro. However, CsA is also a potent inhibitor of calcineurin, a phosphatase that may participate in cell death. Therefore, we tested the hypothesis that calcineurin regulates TNF cytotoxicity in rat hepatoma cells (FTO2B). TNF-treated FTO2B cells appeared apoptotic by DNA fragmentation, nuclear condensation, annexin V binding, and caspase activation. We studied two calcineurin inhibitors, CsA and FK506, and found that each potently inhibited TNF cytotoxicity. Western blot demonstrated calcineurin in FTO2B homogenates. In a model of mitochondrial permeability transition (MPT), we found that CsA prevented MPT and cytochrome *c* release, while FK506 inhibited neither. In summary, we present evidence that calcineurin participates in an apoptotic death pathway activated by TNF. CsA may oppose programmed cell death by inhibiting calcineurin activity and/or inhibiting mitochondrial signaling.

Key words: Calcineurin; Apoptosis; Mitochondrion; Cytochrome *c*; Cyclosporin A; FK506

1. Introduction

Tumor necrosis factor (TNF) binds to receptors on the cell surface that can activate death pathways. Cytoprotective responses limit the susceptibility of most normal and neoplastic cells to TNF in a complex, transcription-dependent process, mediated in part by transcription factors AP-1 and NFκB [1,2]. Inhibitors of transcription or protein synthesis (e.g. actinomycin D (Act D) or cycloheximide) sensitize resistant cells to TNF and allow unopposed activation of proteins in the death pathway.

Aggregation of TNF receptors on the cell surface activates caspases, a family of constitutive proteins with a central role in death pathways [3]. Caspase activation occurs when procaspases interact with receptor death domains. Receptor-associated caspases (e.g. caspase-8) then activate downstream procaspases by proteolytic cleavage. These downstream caspases cleave both structural and regulatory proteins, leading to apoptotic disassembly of the cell. Caspase activation does not require transcription and synthesis of new protein, but may be regulated by compartmentalization and by inhibitory proteins. Inhibition of caspases with synthetic oligopeptides (Z-Val-Ala-Asp-CH_2F (zVAD.fmk)) and naturally occurring viral products (CrmA, baculovirus) opposes apoptosis due to oxidative stress, irradiation, TNF, Fas ligand, and staurosporine.

Recently, a role for mitochondria in the regulation of cell death has become apparent. In the highly TNF-sensitive L929 cell line, release of reactive oxygen species from mitochondria occurs early after TNF exposure, and antioxidant strategies promote cell survival [4]. In apoptosis in a variety of other systems, cytochrome *c*, apoptosis-activating factor, and procaspase-3 are released from mitochondria and interact with caspases and proteins of the *bcl-2* family to mediate cell death [5–7]. Release of these signaling proteins from mitochondria occurs after permeability pores in the inner mitochondrial membrane open and the organelle swells. Cyclosporin A (CsA) prevents the mitochondrial permeability transition (MPT) in vitro by binding to a cyclophilin-like isomerase in the mitochondrial membrane [8,9]. CsA may oppose TNF cytotoxicity in vivo by inhibiting MPT and release of signaling molecules [10].

In addition to its effects on mitochondria, CsA potently inhibits calcineurin (CN), a widely distributed serine-threonine phosphatase that plays a key role in immune suppression [11,12]. CN has been implicated in calcium-dependent death pathways in thymocytes [13] and neural tissues [14], and may influence mitochondrial death signaling via direct interaction with *bcl-2* [15–17] and dephosphorylation of *bad* [18]. Although caspase activation and mitochondrial signaling appear to have prominent roles in death pathways activated by TNF, the role of CN in TNF cytotoxicity has not been determined. In this report, we present evidence that CN participates in TNF-mediated apoptosis in the rat hepatoma (FTO2B) cells.

2. Materials and methods

2.1. Cytokines, reagents and cells

Recombinant rat TNF-α (specific activity 1.33×10^8 U/mg per L929 bioassay) was purchased from Genzyme. FK506 was generously provided by Fujisawa. zVAD.fmk was purchased from Kamiya Biomedical Co.

FTO2B cells were cultured in DMEM/F-12 supplemented with 10% fetal calf serum, 7.5% (w/v) sodium bicarbonate, 2 mM glutamine, penicillin (100 U/ml) and streptomycin (0.1 mg/ml) at 37°C. Plates were coated with gelatin in Williams E media supplemented with HEPES, glutamine, insulin, penicillin and streptomycin.

2.2. Cytotoxicity assay

Cells were removed from the plates using trypsin, counted on a hemocytometer, and plated at a density of 2.5×10^5 per well on gelatin-coated 12-well plates. Cells were allowed to adhere overnight, and then incubated in fresh media containing Act D (1 μg/ml) or TNF (500 U/ml)+Act D. For all experiments, the following inhibitor con-

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centrations were used: 1 μM CsA, 100 nM FK506 or 20 μM zVAD.fmk. Maximal (100%) killing was determined by adding Triton X-100 (final concentration 0.5%) to cells incubated with Act D. Lactate dehydrogenase (LDH) activity in the supernatant was determined at 0, 12 and 18 h using LDH reagent and measuring the rate of change in absorbance at 340 nm for 6 min in a microplate reader. All experiments were performed in triplicate.

2.3. Western blots

For CN immunoassays, cells were grown in T75 flasks to 2×10^7 cells per flask, and the cells were trypsinized, centrifuged, washed and disrupted with three passes in an Elvejiem–Potter glass homogenizer in 0.5 ml of cold lysis buffer with anti-proteases (150 mM NaCl, 50 mM Tris, pH 7.6, 1% sodium dodecyl sulfate (SDS), 3% Nonidet P-40, 5 mM ethylene diamine tetra-acetic acid (EDTA), 1 mM $MgCl_2$, 2 mM 1,3-dichloroisocoumarin, 2 mM 1,10-phenanthroline, and 0.4 mM E-64). After centrifugation at 10 000 rpm for 10 min, the supernatant was stored at −20°C. Cytosol extracts in lysis buffer were mixed with an equal volume of Laemmli sample buffer with 2% mercaptoethanol. Electrophoresis was performed on 12% polyacrylamide gels with 0.1% SDS using a mini-gel system. Lanes were loaded with 10–25 μg of sample protein and electrophoresed for 1.5 h at 100 V. Proteins were then electrotransferred to nitrocellulose paper, and blocked overnight at 4°C in Tris-buffered saline (TBS) with 0.02% sodium azide and 5% non-fat dry milk. Western blots were then prepared using murine antibody to the native form of CN subunit A and subunit B (Sigma). Membranes were incubated for 1 h at 25°C with primary antibody (1:3000) in TBS/5% milk, washed three times in TBS/0.05% Tween (TTBS), and incubated with biotinylated goat anti-mouse antibody for 1 h at 25°C. After three additional washes with TTBS, the membranes were incubated with avidin–horseradish peroxidase (HRP) (1:3000) and signal was detected by electrochemiluminescence (Pierce). All materials used in the preparation of the Western blots were obtained from Bio-Rad.

For cytochrome *c* Western blots, samples of supernatant from mitochondrial suspensions were collected as described below. After dilution with Laemmli sample buffer (1:1), 25 μl of each sample was loaded on a 12% polyacrylamide gel and electrophoresed for 1.5 h at 100 V. After blocking overnight, membranes were incubated for 1 h at 25°C at mouse anti-cytochrome *c* (Pharmingen) (1:1000). After rinsing, the membrane was incubated for 1 h with anti-mouse IgG–biotin conjugate (Sigma) (1:20 000), rinsed and incubated for 1 h with avidin–HRP conjugate (1:3000). Signal was detected by chemiluminescence (ECL from Amersham).

2.4. DNA electrophoresis

For each experiment, DNA was isolated from 2×10^6 cells using an apoptotic DNA ladder kit (Boehringer Mannheim). Cells were added to DNA binding buffer (6 M guanidine–HCl, 10 mM urea, 10 mM Tris–HCl (pH 4.4), 20% Triton X-100) and applied to a glass fleece filter tube. After washing with 80% ethanol in 20 mM NaCl and 2 mM Tris–HCl (pH 8.5), DNA was eluted in 10 mM Tris–HCl (pH 8.5) and assayed immediately. Samples were added to 30% glycerol and electrophoresed on a 1% agarose gel with ethidium bromide for 1–1.5 h at 75 V. DNA was illuminated with an ultraviolet light source and photographed.

2.5. Nuclear morphology

Cells were fixed in 4% paraformaldehyde at 25°C for 30 min, then washed twice with phosphate-buffered saline (PBS). The cell pellet (approximately 10^6 cells) was then resuspended in 200 μg/ml lysophosphatidyl choline with 5 U of RNase and 10 μg propidium iodide at 37°C for 30 min. Cells were then transferred to slides and examined immediately with a fluorescent microscope (Nikon TE300) using a rhodamine filter.

2.6. Flow cytometry

Cell binding of annexin and permeability to propidium iodide were assessed by flow cytometry in order to assess the extent of apoptosis [19]. In normal cells, phosphatidylserine (PS) is located on the cytoplasmic surface of the cell membrane, but in cells undergoing apoptosis, this phospholipid orientation is reversed. In impermeable cells early in apoptosis, annexin V binds to the PS on the surface of the cell. In cells undergoing necrosis or late in apoptosis, the cell membrane is permeable to the nucleic acid stain propidium iodide, and annexin V binds to PS on the cytoplasmic surface. Fresh cells treated with TNF were removed from the flask, washed with cold PBS and suspended at 10^6 cells/ml in buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4). Cells were then incubated with annexin V conjugated to a fluorophore (Molecular Probes) and propidium iodide (1 μg/ml) at 25°C for 15 min, and then assayed by flow cytometry (FACSCalibur; Becton Dickinson).

2.7. Caspase activity

Determinations of caspase activity were carried out in 96-well plates using cell lysate from 2×10^6 cells for each measurement. Caspase activation was measured in cells treated with TNF+Act D or Act D alone for 6 h. Lysis buffer, reaction buffer and colorimetric substrates for caspases were obtained from R&D Systems. For caspase-3 determinations, DEVD-pNA (caspase substrate conjugated to *p*-nitroaniline) was added to the reaction mixture, and after a 2 h incubation at 37°C, absorbance was measured at 405 nm. Caspase-8 activity was measured by substituting the colorimetric substrate IETD-pNA for DEVD. For determination of caspase inhibition, cells were incubated with zVAD.fmk and TNF+Act D for 6 h prior to preparation of the cell lysate.

2.8. MPT and cytochrome c release

Rat liver mitochondria were isolated from Sprague–Dawley rats (300–400 g) using a density gradient centrifugation as previously described [20]. The permeability transition was detected by measuring absorbance at 540 nm (A_{540}) of mitochondria (0.3 mg/ml) suspended in MPT buffer (100 mM KCl, 75 mM mannitol, 25 mM sucrose, 5 mM Tris–phosphate (pH 7.4), 10 mM Tris–HCl (pH 7.4), 0.05 mM EDTA and 5 mM succinate) [21]. The permeability transition was activated by the addition of 300 μM $CaCl_2$ to suspensions of control mitochondria or to suspensions containing 1–50 μM CsA or 100 nM to 50 μM FK506. To determine release of cytochrome *c*, freshly isolated rat liver mitochondria (respiratory control ratio of 4–5) were suspended in MPT buffer at 5 mg/ml. 5 min after addition of $CaCl_2$ and/or inhibitors (1 μM CsA; 100 nM FK506 ± 2 μg/ml FKBP), the mitochondrial suspensions were centrifuged (16 000 × *g* for 1 min) and supernatants stored at −80°C.

2.9. Statistical analysis

Statistical analyses were performed using the paired or unpaired Student's *t* test for comparison of means or analysis of variance (ANOVA) for repeated measures.

3. Results

3.1. Characterization of cell death

FTO2B cells were incubated with TNF+Act D for 12 h, permeabilized and stained with propidium iodide in the presence of RNase. Examination of treated cells by fluorescent microscopy demonstrated discrete clumping of nuclear chromatin in the majority (Fig. 1A). Staining of control cells (treated with Act D alone) demonstrated diffuse, homogeneous fluorescence of the nuclei, with rare clumping of chroma-

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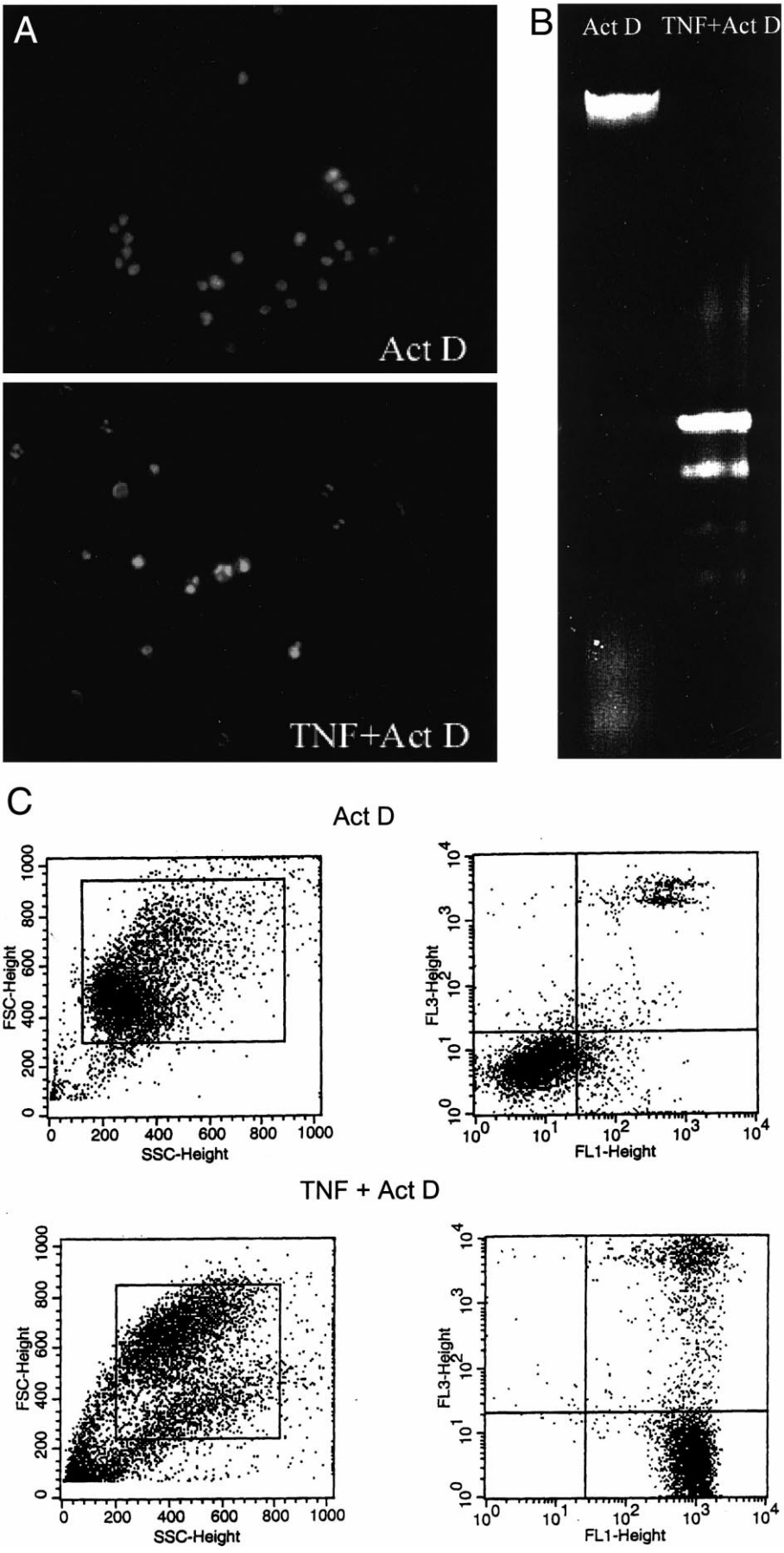
Fig. 1. Characterization of cell death. A: Nuclear morphology. Propidium iodide stain demonstrates altered nuclear morphology of FTO2B cells after 12 h exposure to Act D (1 μg/ml) (top) or TNF (500 U/ml)+Act D (1 μg/ml) (bottom). B: DNA fragmentation. Electrophoresis of DNA extracted from FTO2B cells after 24 h exposure to TNF+Act D demonstrates DNA fragmentation compared with control (treated with Act D alone). C: Annexin binding. Flow cytometry of cells incubated with annexin V and propidium iodide after incubation with Act D (top) or TNF+Act D for 12 h (bottom). Control cells demonstrate impermeability to propidium iodide (*y* axis, FL3) and no significant annexin binding (*x* axis, FL1), while treated cells are impermeable to propidium iodide and bind annexin.


A
Act D
TNF+Act D
B
Act D
TNF+Act D
C
Act D
FSC-Height
SSC-Height
FL3-Height
FL1-Height
TNF + Act D
FSC-Height
SSC-Height
FL3-Height
FL1-Height

tin. The morphological changes after exposure to TNF were consistent with chromatin condensation and formation of apoptotic bodies.

DNA isolated from cells after incubation with TNF+Act D for 24 h was characterized by electrophoresis. The gel pattern observed was consistent with apoptosis, in which DNA cleavage by endonucleases results in fragments in multiples of 180–200 bp (Fig. 1B). Control cells incubated with Act D demonstrated genomic DNA of high molecular weight, with no evidence of DNA fragmentation.

Cells treated with TNF+Act D for 12 h were incubated with annexin V and propidium iodide. We found that ~70% of FTO2B cells treated with TNF+Act D bound annexin V but not propidium iodide, consistent with apoptosis. The remaining cells (~30%) demonstrated binding of both annexin V and propidium iodide, consistent with either necrosis or late apoptosis (Fig. 1C).

Assay of caspase activation demonstrated increases in caspase-3 and caspase-8 after treatment with TNF+Act D within 6 h (Fig. 2). This activation occurred before any significant cell death was detected by LDH release. Incubation of cells with the non-selective caspase inhibitor zVAD.fmk inhibited caspase-3 and caspase-8 activity.

3.2. Effect of CN inhibition on TNF cytotoxicity

Incubation of FTO2B cells with 1 μM CsA or 100 nM FK506 during treatment with TNF+Act D potently inhibited cytotoxicity (Fig. 3A). Treatment with Act D, CsA or FK506 alone did not cause release of LDH from cells over the 18 h incubation (data not shown). The intact CN protein dimer (~80 kDa) was present in FTO2B cells by Western blot for CN subunit A (Fig. 3B) and subunit B (Fig. 3C).

3.3. Effect of CN inhibitors on the MPT

Well-coupled, isolated rat liver mitochondria were induced to undergo the permeability transition by adding calcium to a mitochondrial suspension, and then following the change in absorbance (A_{540}) for 20 min. CsA but not FK506 (±FKBP) inhibited the calcium-dependent permeability transition in vitro (Fig. 4A). Western blot demonstrated potent inhibition of cytochrome *c* release from isolated rat liver mitochondria by CsA, but no effect of FK506 on cytochrome *c* release (Fig. 4B).

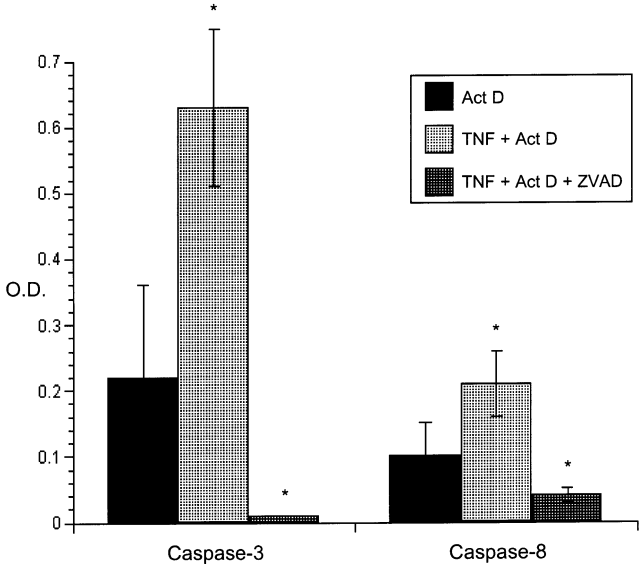


Fig. 2. Caspase activation in TNF-treated FTO2B cells. Caspase-3 and caspase-8 activity increase in FTO2B cells treated with TNF+Act D for 6 h, while both are inhibited by zVAD.fmk (*$P < 0.05$).

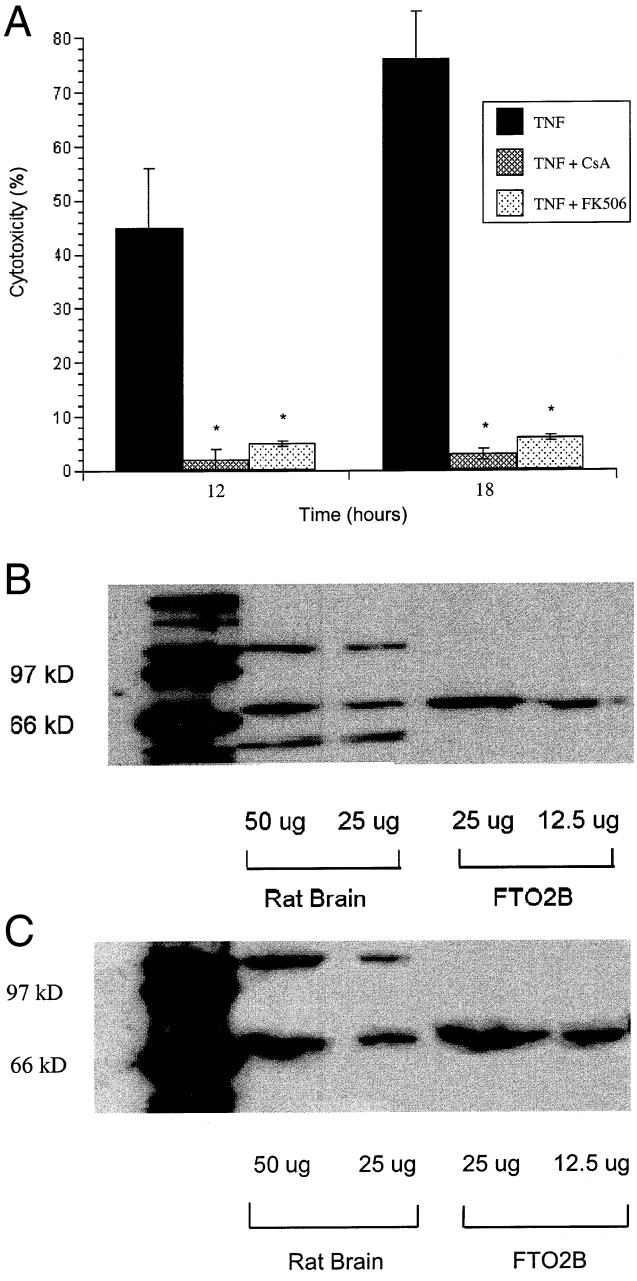


Fig. 3. Effect of CN inhibition on TNF cytotoxicity. A: Cytotoxicity determined by LDH release for FTO2B cells treated with TNF+Act D is inhibited by cyclosporin A or FK506 (*$P < 0.05$ by ANOVA). B: Western blot for CN subunit A demonstrates CN protein in cytosol of untreated FTO2B cells (positive control: rat brain). C: Western blot for CN subunit B confirms CN protein in FTO2B cells, migrating at a molecular weight consistent with the intact dimer.

4. Discussion

In the FTO2B cell line, TNF caused death with features typical of apoptosis: nuclear condensation, DNA fragmenta-

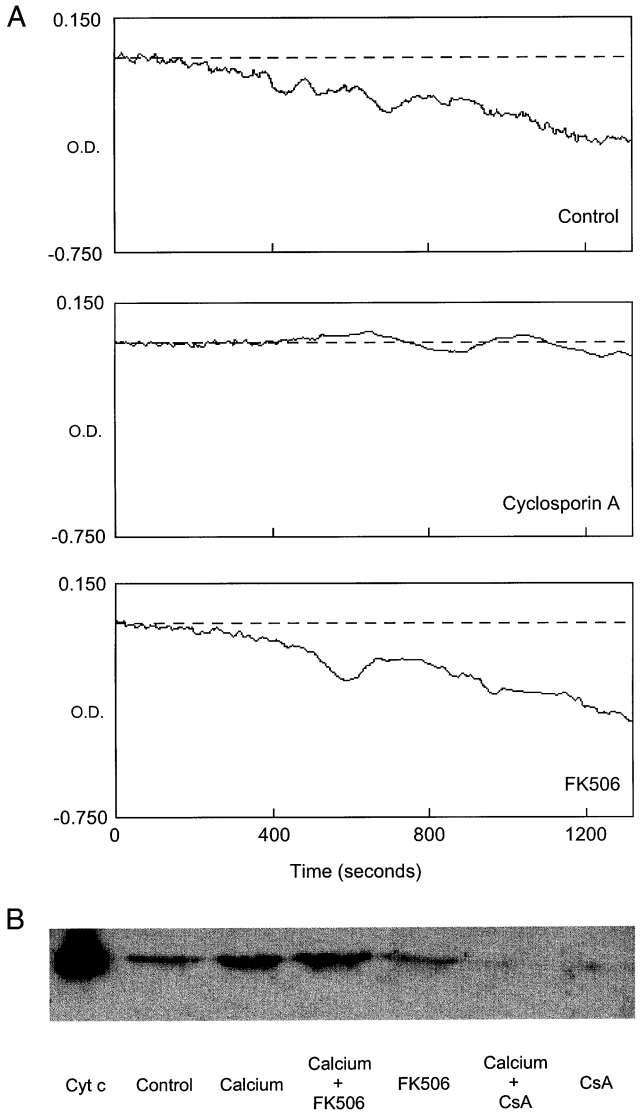


Fig. 4. Effect of CN inhibitors on MPT and cytochrome *c* release. A: Change in absorbance of mitochondrial suspension (0.3 mg/ml) over time at 540 nm after addition of calcium chloride is inhibited by 10 μM CsA but not 10 μM FK506. Addition of FKBP (1 μg/ml) did not affect MPT (data not shown). B: Release of cytochrome *c* from isolated rat liver mitochondria is inhibited by 1 μM CsA, but not 100 nM FK506.

tion, annexin binding, and activation of the caspase pathway. TNF cytotoxicity in several cell types and in vitro systems proceeds to death via a pathway consistent with apoptosis [22], however, TNF cytotoxicity may also produce death with features of necrosis (e.g. L929 cells, fibroblasts [23]). In both apoptosis and necrosis, death signaling via the MPT may be important. The term 'necroptosis' has been proposed to emphasize the potential regulatory overlap in these processes [24].

Potent inhibition of TNF cytotoxicity in hepatoma cells by two CN inhibitors, CsA and FK506, suggests that CN participates in a death pathway activated by TNF. The protection observed with FK506 does not appear to be due to inhibition of mitochondrial signaling, as FK506 was without effect on the MPT and cytochrome *c* release in rat liver mitochondria. In addition, no effect of FK506 on the MPT was observed in mitochondria isolated from rat kidney [25]. However, we did not characterize mitochondrial signaling in intact hepatoma cells, and our findings do not exclude a role for mitochondria in this pathway.

CN is a calcium-dependent phosphatase present in the cytosol as a heterodimer, with a 58–64 kDa catalytic and calmodulin binding portion (subunit A), and a 19 kDa calcium binding regulatory portion (subunit B) [26]. An iron–zinc metal center in the active site of subunit A is required for the phosphatase activity, and oxidation of the iron reversibly inhibits activity. The original substrate specificity described for CN included dephosphorylation of the cytosolic transcription regulator, nuclear factor activated T cells (NFAT). Dephosphorylation of NFAT and its translocation to the nucleus is followed by induction of several cytokines, including interleukin-2, interleukin-4 and TNF. Because cyclosporin A and FK506 potently inhibit CN activity and NFAT-mediated immune events, they have become critical immunosuppressant therapies in organ transplantation.

The clinical use of cyclosporin A and FK506 for immunosuppression in humans is associated with an increased risk for malignancies, including squamous cell cancer, Kaposi's sarcoma, cervical carcinoma, and lymphoproliferative disorder. Clonal expansion of B cells in lymphoproliferative disorder has been attributed to viral infection in the setting of depressed T cell function, while decreased immune surveillance and increased exposure to TGF-β may contribute to solid tumor proliferation [27]. Altered regulation of cell death may also participate in neoplasia, and a role for CN in the regulation of cell death has been proposed for neurons and immune cells.

In cerebellar granule cells treated in culture with glutamate, apoptotic death was inhibited by either CsA or FK506 [14]. In cultured retinal neurons, brief exposure to glutamate resulted in cell death that was prevented by inhibitors of CN activity [28]. Thymocytes treated with thapsigargin to raise intracellular calcium levels are induced to undergo DNA fragmentation, and this fragmentation is prevented by treatment with CsA or FK506 [13]. Apoptosis in a B cell line from human Burkitt's lymphoma induced by anti-IgM antibody was also prevented by treatment with FK506 [29]. CN inhibitors also prevent apoptosis in a T cell hybridoma line, suggesting that CN is a critical mediator of T cell receptor/CD3 death signaling [30,31].

Important interactions may occur between CN and members of the *bcl-2* family of pro- and anti-apoptotic proteins. In baby hamster kidney (BHK) cells transfected with DNA encoding CN subunits A and B, CN expression increased sensitivity to calcium-induced death [15]. Co-expression of anti-apoptotic protein *bcl-2* inhibited CN-dependent apoptosis in this model, and direct interaction of *bcl-2* with the cytosolic CN was observed. The pro-apoptotic protein *bax* interfered with co-localization of CN and *bcl-2* [17]. *Bad*, another pro-apoptotic member of the *bcl-2* family, appears to be a substrate for the phosphatase activity of CN [18]. Interactions between CN and the *bcl-2* family described in the transfected BHK cells suggest a mechanism for regulation of mitochondrial death signaling by CN activity.

In summary, we report that TNF-mediated death in rat hepatoma cells is regulated by CN. In this model of apoptosis, treatment with CsA can oppose cell death via CN inhibition, and this effect should be considered in studies of the effect of

CsA on the MPT. Finally, in organ transplant recipients treated with CsA or FK506, inhibition of CN-dependent death pathways may prevent apoptosis and result in progression of lymphatic and epithelial tumors.

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