Analysis of Fumonisin B_1-Induced Apoptosis

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Fumonisins are mycotoxins produced by *Fusarium moniliforme,* a prevalent fungus that infects corn and other cereal grains. Fumonisin B_1 (FB_1) is the most common mycotoxin produced by *F. moniliforme*, suggesting it has toxicologic significance. The structure of FB_1 resembles sphingoid bases, and it inhibits ceramide synthase. Because sphingoid bases regulate cell growth, differentiation, transformation, and apoptosis, it is not surprising to find that FB_1 can alter growth of certain mammalian cells. Previous studies concluded FB_1-induced apoptosis, or cell cycle arrest, in African green monkey kidney fibroblasts (CV-1). In this study we have identified genes that inhibit FB_1-induced apoptosis in CV-1 cells and two mouse embryo fibroblasts (MEF). A baculovirus gene, inhibitor of apoptosis (CpIAP), protected these cells from apoptosis. CpIAP blocks apoptosis induced by the tumor necrosis factor (TNF) pathway as well as other mechanisms. Further support for the involvement of the TNF signal transduction pathway in FB_1-induced apoptosis was the cleavage of caspase 8. Inhibition of caspases by the baculovirus gene *p35* also inhibited FB_1-induced apoptosis. The tumor suppressor gene *p53* was not required for FB_1-induced apoptosis because $p53^{-/-}$ MEF undergo apoptosis following FB_1 treatment. Furthermore, *Bcl-2* was not an effective inhibitor of FB_1-induced apoptosis in CV-1 cells or $p53^{+/+}$ MEF. In summary, these results provide new information to help understand the mechanism by which FB_1 induces apoptosis. *Key words:* apoptosis, fumonisin B_1, mycotoxins, signal transduction, tumor necrosis factor. — *Environ Health Perspect* 109(suppl 2):315–320 (2001).
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Introduction

Fusarium moniliforme is a significant plant pathogen that primarily infects corn but can also infect rice and wheat [reviewed in (*1–7*)]. The fungus is widespread and produces a variety of mycotoxins; the major class is called fumonisin. Several subtypes of fumonisin have been identified (A, B, or C), and all these compounds resemble sphingolipids [reviewed in (*8–10*)]. FB_1 inhibits sphingosine *N*-acetyltransferase (ceramide synthase) and thus blocks sphingolipid biosynthesis. FB_1 and FB_2 are of toxicologic significance because they are present at high concentrations on diseased plants and are frequently detected on healthy corn plants (*11*).

Ingestion of moldy corn infected by *F. moniliforme* or closely related fungi is linked to a higher incidence of primary liver cancer (*12*) and esophageal cancer in regions of South Africa and China (*13*). In rats fed a diet containing 50 mg/kg FB_1, primary hepatocellular carcinoma occurred in two thirds of the animals (*14–16*). FB_1 is also a potent tumor promoter in rat liver after initiation with diethylnitrosamine (*17*). Cancer-promoting activity has occurred at levels that did not induce liver or kidney toxicity and, furthermore, did not initiate cancer.

Culture material from *F. moniliforme* containing FB_1 induces equine leukoencephalomalacia and nephrotoxicity (*18*). Purified preparations of FB_1 are also hepatotoxic (*19*), cause porcine pulmonary edema (*20*), and are nephrotoxic to several animal species (*21–23*). *In vivo* studies have demonstrated that FB_1 induces apoptosis in rat kidney and liver (*24–26*). FB_1 also induces apoptosis of several different mammalian cell types (*26–29*), demonstrating that treatment can lead directly to apoptosis. A previous study demonstrated that neoplastic African green monkey kidney cells (COS-7) are resistant to the apoptotic and antiproliferative effects of FB_1 compared to their normal counterparts, African green monkey kidney cells (CV-1) (*29,30*). Murine or human leukemia cells do not undergo apoptosis when treated with FB_1 (*30,31*). We have also found that other cell lines derived from human tumors are resistant to the effects of FB_1 (data not shown). Taken together, these studies suggested that transformed cells, in general, are resistant to the effects of FB_1. Because the ability of cells to escape apoptosis plays a critical role in the development of cancer, the ability of FB_1 to induce apoptosis may select for cells resistant to apoptosis. It is hypothesized that these cells would have altered growth capabilities.

The goal of this study was to characterize FB_1-induced apoptosis. A previous study demonstrated that cyclin-dependent kinase (cdk) activity increased slightly after 3 hr of FB_1 treatment but then declined (*30*). This is significant because cdk activity transiently increases during the early stages of apoptosis (*32*). Further studies confirmed that cdk2 activity transiently increased after FB_1 treatment. Several pathways have been identified that result in programmed cell death or apoptosis [reviewed in (*33*)]. A quantitative assay for apoptosis was used to identify specific genes that interfere with FB_1-induced apoptosis. This assay indicated that a baculovirus gene (CpIAP) inhibited FB_1-induced apoptosis. Because CpIAP inhibits tumor necrosis factor (TNF)-induced apoptosis, we further examined other factors involved in TNF signal transduction pathways. A caspase involved with TNF-induced apoptosis (caspase 8) was cleaved and activated within 2 hr after treatment. In contrast, nuclear factor kappa-B (NF-κB), a transcription factor downstream of the TNF receptor (TNFR) but independent of the apoptotic pathway was not activated following FB_1 treatment. Taken together, this study suggested that the TNF signal transduction pathway (directly or indirectly) plays a role in FB_1-induced apoptosis. Further studies will be necessary to prove that the TNFR or downstream factors are directly affected by FB_1.

Materials and Methods

Cells Lines and Media

CV-1 cells were split at 1:5 ratio every 5 days. Mouse embryo fibroblasts (MEF) with a disrupted p53 gene ($p53^{-/-}$) were obtained from E. Lee (University of Texas Southwestern Medical Center, San Antonio, Texas, USA) and T. Jacks (Massachusetts Institute of Technology, Cambridge, MA, USA). Normal MEF were prepared from Balb/C mice. MEF were split 5–7 times and then discarded. Cells were grown in Earle's modified Eagle's medium supplemented with 5% fetal bovine serum (FBS), penicillin (10 U/mL), and streptomycin (100 μg/mL) and were plated at a density of 5×10^5 cells/100-mm plastic dish.

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Fumonisin B_1

FB_1 was obtained from R. Eppley (U.S. Food and Drug Administration, Washington, DC, USA) (> 99% pure) and was dissolved in calcium and magnesium-free, phosphate-buffered saline (PBS) (80 mM Na_2HPO_4; 20 mM NaH_2PO_4; 100 mM NaCl, pH 7.5). Three hours after the cells were plated, FB_1 was added to the media at a concentration of 5 or 25 µM.

Bluo-gal Co-Transfection and Analysis of Cell Death

Cells were plated at a density of 2×10^5/well in 6-well plastic plates (35 mm/well) 12–16 hr prior to the transfection. Cells were then transfected with plasmid DNA containing the β-galactosidase gene [cytomegalovirus (CMV-β-gal)] by the calcium phosphate method (*34*). Samples were co-transfected with a mammalian expression plasmid that influences cell growth or apoptosis. A description of these plasmids is presented below. For each 35-mm well, 250 µL calcium phosphate solution (125 mM $CaCl_2$; 25 mM HEPES–NaOH, pH 7.1; 0.75 mM Na_2HPO_4–NaH_2PO_4, pH 7.0; 125 mM NaCl) and plasmid DNA mix were added to the cells. Five hours after transfection, cells were shocked with PBS in 20% glycerol for 4 min, then washed with PBS twice. Fresh medium containing 5% fetal calf serum and 5 mM sodium butyrate was added to the cells. Cells were incubated at 37°C for 16 hr, then passaged at a 1:5 ratio onto a 24-well dish. These cells were treated with 25 µM FB_1 or 20 µM C_6-ceramide (37°C for 48 hr), and β-gal expression was analyzed as described previously (*27,35*). Briefly, cells were rinsed with PBS, fixed with 2% formaldehyde, 0.2% glutaraldehyde in PBS for 5 min, then washed twice with PBS. Fixed cells were stained for 6–24 hr with 0.1% Bluo-gal (Gibco, Rockville, MD, USA) in a PBS solution containing 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 2 mM $MgCl_2$. After being rinsed in PBS, positive cells were observed microscopically and the number of stained blue cells counted. At least five fields per plate were counted, and the average number of cells per field was calculated.

Plasmids

pCMVCpIAP and pcDNA3/p35 are plasmids that contain baculovirus anti-apoptotic genes (*36*); these plasmids were obtained from L. Miller (University of Georgia, Athens, GA, USA). pcDNA/crmA (*37*) encodes a cowpox virus protein inhibitor of caspases and was obtained from L. Lou (University of Michigan Medical School, Ann Arbor, MI, USA). The adenovirus E1A or E1B 55-kDa encode proteins that bind Rb or p53, respectively, and were obtained from E. White (Rutgers University, Piscataway, NJ, USA). The expression vector that encodes Bcl-2 was obtained from J. Reed (La Jolla Cancer Research Center, La Jolla, CA, USA). CMV-β-gal was purchased from Clontech (Palo Alto, CA, USA). Human immunodeficiency virus (HIV) long terminal repeat (LTR) and HIVΔNFkB were obtained form C. Wood (University of Nebraska, Lincoln, NE, USA). Plasmid preparations were obtained by cesium chloride purification (*38*).

Nuclear DNA Fragmentation Analysis

Cell cultures were treated with 5 µM FB_1 for 6, 12, 24, or 48 hr. Cells were then collected with a plastic cell scraper, suspended in TKMC buffer (10 mM Tris, pH 7.5; 10 mM KCl; 1 mM $MgCl_2$; 5 mM $CaCl_2$) containing 0.5% TX-100. Cells were lysed by treatment with 150 µg/mL of proteinase K, 100 mg RNAse A, and 0.1% sodium dodecyl sulfate (SDS). Genomic DNA was extracted with a phenol–chloroform–isoamyl alcohol solution (50:49:1). DNA samples were electrophoresed in a 1.5% agarose gel using TBE (0.1 M Tris, 0.1 M boric acid, 0.025 M EDTA, pH 8.3). DNA in the agarose gel was denatured with 1.5 M NaCl, 0.5 M NaOH for 30 min at room temperature, then neutralized by incubating with 3.0 M NaCl and 0.5 M Tris, pH 5.2, for 25 min. After the neutralization step was repeated, gels were rinsed in 2× SSC) (20 × SSC is 3 M NaCl and 0.3 M citric acid) and blotted onto a nylon membrane (Hy Bond+, Amersham). The DNA transferred to the membrane was hybridized with a monkey genomic DNA probe. The probe was radiolabeled by random priming method with ^{32}P by an enzymatic reaction catalyzed by the Klenow enzyme (New England Biolabs, Beverly, MA, USA). Prehybridization was performed at 65°C in 5× SSPE (0.75 M NaCl, 0.05 M sodium phosphate, 0.005 M EDTA), 0.5% bovine serum albumin, 0.5% polyvinylpyrrolidone and 1% SDS for 2 hr. Hybridization was carried out for 6–18 hr at 65°C by using 2×10^7 dpm of radiolabeled probe diluted in hybridization buffer. Filters were washed 3 times at room temperature and once at 65°C for 30 min in 1× SSPE, 1% SDS. Filters were then dried and exposed to film.

Measurement of Cyclin-Dependent Kinase Activity

Dishes were washed with PBS and cells were collected, and suspended in 500 µL of lysis buffer (20 mM HEPES, pH 7.9; 0.4 M NaCl; 1 mM EDTA; 10 mM dithiothreitol; 1 mM phenylmethylsulfonyl fluoride; 25% glycerol). Cell lysate was kept on ice for 20 min and lysed by two freeze/thaw cycles. Cells were centrifuged for 10 min at 12,000 rpm, and the supernatant was kept at –80°C. Immunoprecipitations were performed with 150 µg cell lysates for 3–4 hr at 4°C; 1 µg of antibody was used. Immunocomplexes were precipitated with protein A-Sepharose beads (Sigma, St. Louis, MO, USA) and washed 4 times with washing buffer (10 mM Tris–HCl, pH 8.0; 50 mM NaCl; 1 mM EDTA; 0.5% nonidet P40) (*30*). Immunocomplexes were washed in 1× kinase buffer and the pellet was

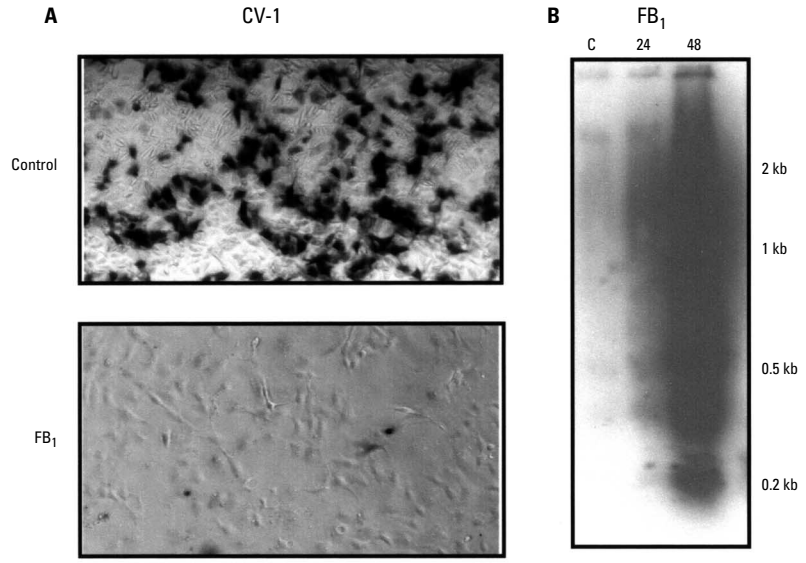

Figure 1. Induction of apoptosis by FB_1. (*A*) CV-1 cells were transfected with plasmid pCMV-β-gal. After transfection, cells were treated with 25 µM FB_1 for 48 hr, fixed, and stained with Bluo-gal for 24 hr. Cultures treated with PBS are designated as controls. Magnification ×100. (*B*) CV-1 cells were treated with 25 µM FB_1 for 24 or 48 hr. High molecular weight DNA was prepared from CV-1 cells and electrophoresed on a 2% agarose gel; Southern blot analysis was performed using monkey DNA as a probe. Cultures not treated with FB_1 are designated as "C." Molecular weight markers are shown to the right.

suspended in 10 µL of the same buffer (10× kinase buffer: 500 mM Tris, 250 mM Mg acetate, 25 mM EDTA). The kinase reaction contained 10 µCi γ-^{32}P-adenosine triphosphate (ATP), 0.1 M ATP, 2.7 µg of histone H1, and 100 µg crude extract diluted in 1× kinase buffer. After 30 min at 30°C, the reaction was stopped by addition of 25 µL of SDS–polyacrylamide gel electrophoresis (PAGE) loading buffer. Reaction products were analyzed by SDS–PAGE, and the gel was dried and autoradiographed. Kinase activity was measured with a PhosphorImager instrument (Molecular Dynamics, Sunnyvale, CA, USA).

Analysis of Chloramphenicol Acetyltransferase Activity

CV-1 cells were transfected with the designated plasmids by calcium phosphate transfection as described previously (*27,34,39*). Following transfection, cultures were treated with 5 µM FB_1. Forty hours after transfection, cells were washed in PBS and a cell-free lysate was prepared by three freeze–thaw cycles in 0.25 M Tris (pH 7.8). Chlorophenicol acetyltranseferase (CAT) enzymatic activity was measured as described previously (*39*). After thin-layer chromatography, the amount of ^{14}C-chloramphenicol (CM), acetylated or unacetylated, was measured with a PhosphorImager device.

Statistical Analysis

Statistical analyses were performed using Instat (Sigma Plot, Chicago, IL, USA), a personal computer software program. Results were considered statistically significant when the p value was < 0.05.

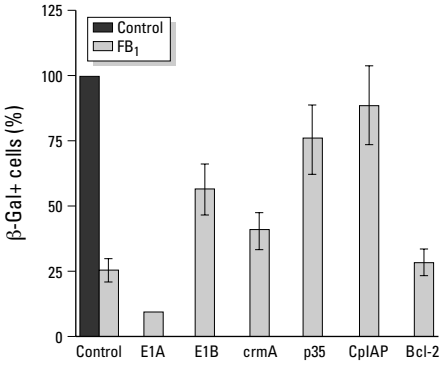

Figure 2. Interference of FB_1-induced apoptosis in CV-1 cells. CV-1 cells were co-transfected with plasmid pCMV-β-gal (2 µg DNA) and plasmids expressing the indicated apoptotic regulatory proteins (2 µg of the indicated plasmid). After transfection, cells were treated with 25 µM FB_1 for 48 hr, fixed, and stained with Bluogal for 24 hr. The number of β-gal+ cells in five fields was determined. The percentage of β-gal+ colonies was normalized based on the number of blue cells in the untreated control. The data in this study were derived from five independent studies. The error bars show the standard error of the mean. The differences between E1B, p35, crmA, or CpIAP and the control are significant ($p < 0.05$).

Results

Identification of Genes That Inhibit Fumonisin B_1-Induced Apoptosis

By using a battery of well-accepted assays that identify apoptotic cells, several independent studies have concluded that FB_1 induces apoptosis in CV-1 cells (*29*) and human cells (*26–29*). To identify genes that regulate apoptosis, we used an assay based on co-transfecting cells with a β-gal expression plasmid (pCMV-β-gal) and a gene that regulates apoptosis (*35,40,41*). If a gene induces apoptosis, the number of β-gal+ cells decrease. Conversely, anti-apoptotic genes increase the number of β-gal+ cells following induction of apoptosis, relative to controls. FB_1 consistently reduced the number of β-gal+ cells in CV-1 cells (Figure 1A). Reduction of β-gal+ cells correlated with apoptosis because nucleosome-length DNA and higher molecular weight forms of degraded DNA accumulated 24 or 48 hr after treatment (Figure 1B, lanes 24 or 48). As expected, untreated CV-1 cells contained low levels of degraded DNA (Figure 1B, lane C).

In CV-1 cells, the baculovirus inhibitor of apoptosis (CpIAP) efficiently inhibited FB_1-induced apoptosis (Figure 2). The *Bcl-2* gene did not effectively inhibit apoptosis by FB_1. Two caspase inhibitors, crmA and p35, appeared to give slightly different results. p35 consistently inhibited FB_1-induced apoptosis slightly better than crmA in CV-1 cells. The adenovirus E1B gene encodes a 55-kDa protein that binds and inactivates the p53 tumor-suppressor protein [reviewed in (*33*)]. E1B protected cells from apoptosis by FB_1 but not as efficiently as CpIAP. As expected, the adenovirus E1A gene induced apoptosis and FB_1 had little or no effect on this process. In summary, these studies indicated that CpIAP efficiently inhibited apoptosis by FB_1.

FB_1-Induced Apoptosis Does Not Require p53

To determine whether p53 was required for FB_1-induced apoptosis, studies were performed in MEF derived from $p53^{-/-}$ mice and subsequently compared to results obtained in $p53^{+/+}$ MEF. The studies presented in Figure 2 indicated that E1B did not completely prevent apoptosis by FB_1, suggesting p53 was important but not required. We were particularly interested in determining whether p53 was required for FB_1-induced apoptosis because of the importance p53 plays in apoptosis and cell growth. $p53^{-/-}$ cells are ideal for these studies because they are low-passage cells that lack only p53, whereas many established cells lacking p53 have additional unidentified mutations. Treatment of $p53^{-/-}$ cells with 25 µM FB_1 for 24 hr led to the appearance of the typical DNA ladder characteristic of apoptosis (Figure 3A). When lower concentrations of FB_1 were used (5 µM), apoptosis occurred but at a lower efficiency (data not shown). The β-gal transfection assay also demonstrated that 25 µM FB_1 reduced the number of β-gal+ cells (Figure 3B), and CpIAP protected $p53^{-/-}$ cells from apoptosis. p35 and CpIAP protected both MEF more efficiently from apoptosis relative to crmA. Bcl-2 protected $p53^{-/-}$ MEF more effectively than $p53^{+/+}$ MEF from FB_1-induced apoptosis. Although p53 was not absolutely required for apoptosis induced by FB_1, it appeared to have an effect on the ability of Bcl-2 to inhibit apoptosis.

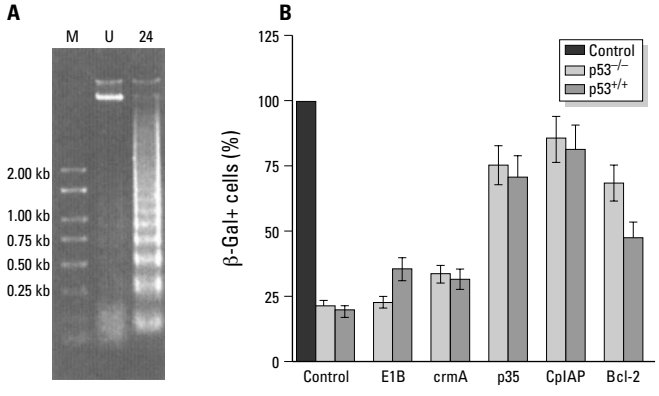

Figure 3. Analysis of apoptosis in MEF after FB_1 treatment. (*A*) Genomic DNA was prepared from $p53^{-/-}$ MEF after treatment with 25 µM FB_1 for 24 hr. As a control, DNA was prepared from untreated cells (U). DNA was separated on 2% agarose gels, and the DNA was stained with ethidium bromide. The position and size (Kb) of the marker DNA (M) is indicated. (*B*) $p53^{-/-}$ or $p53^{+/+}$ MEF were co-transfected with pCMV-β-gal (2 µg DNA) and a plasmid expressing the indicated apoptotic regulatory proteins (2 µg). Cultures were treated with 25 uM FB_1 after transfection. The results are the mean of five independent experiments. The arrow bars show the standard error of the mean. The differences between p35, Bcl-2, or CpIAP and the control are significant ($p < 0.05$).

Analysis of the TNF Pathway after FB_1 Treatment

CpIAP interferes with TNF/Fas induced apoptosis by binding to caspase 3, a direct target of caspase 8 and 9 [reviewed in (*42*); summarized in Figure 4]. This suggested the ability of CpIAP to efficiently inhibit FB_1 induced apoptosis was because the TNF signaling pathway was involved. However, CpIAP also binds directly to caspase 9 and 7, suggesting that FB_1-induced apoptosis was not directly related to the TNF/Fas signaling pathway. Caspases are important for apoptosis because they degrade proteins necessary for cell survival. Because caspase 8 is required for TNF/Fas-induced apoptosis and it must be cleaved to be activated (*43*), we tested whether caspase 8 was cleaved after FB_1 treatment. An antibody that recognizes intact and cleaved caspase 8 was used for this study. At 2 and 6 hr after FB_1 treatment, a slight increase in the amount of cleaved caspase 8 (10-kDa band) was observed in CV-1 cells treated with 25 μM FB_1 (Figure 5). Although similar levels of uncleaved caspase 8 were seen in control cultures, the amount of the 10-kDa band was lower.

NF-κB activation can also occur following TNFR activation, but different accessory proteins are involved [(*42*); summarized in Figure 4]. In contrast to caspase 8 cleavage, activation of NF-κB appears to be related to cell survival (*41*). Consequently, we tested whether FB_1 treatment had any effect on NF-κB activity. For this study, we used the HIVLTR and an NF-κB mutant (HIVΔNFκB). At 3, 24, or 48 hr after FB_1 treatment, neither plasmid had higher promoter activity (Figure 6). In contrast, infection of cells transfected with HIV LTR with herpes simplex virus type 1 led to higher levels of HIV LTR promoter activity, as described previously (*44*). Under identical conditions we previously demonstrated that the promoter regulating the cdk inhibitor (p21) was activated by FB_1 treatment (*39*). In summary, caspase 8 but not NF-κB was activated by FB_1 treatment.

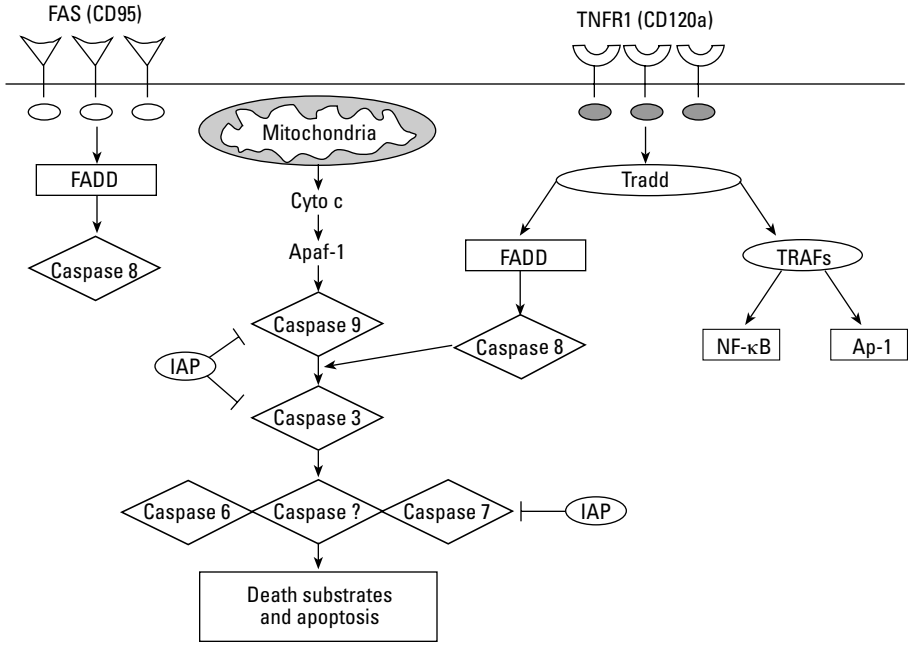

Figure 4. Schematic of TNF/Fas signal transduction pathway. See text for details.

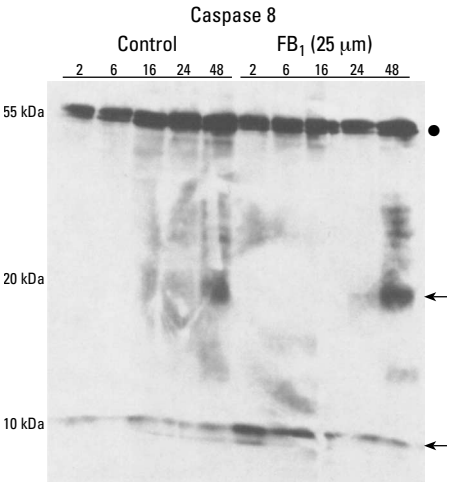

Figure 5. Cleavage of caspase 8 after FB_1 treatment. CV-1 cells were treated with FB_1 for the designated time, whole cell extracts prepared, and 50 μg protein run on a 12% SDS–PAGE (*30*). Western blots were probed with an antibody directed against caspase 8 (PharMingen catalog 66231A, BD PharMingen, San Diego, CA, USA). The circle represents uncleaved caspase 8, the arrow at 20 kDa a putative breakdown product, and the block arrow the 10-kDa caspase 8 proteolytic breakdown product. This Western blot is representative of three independent experiments.

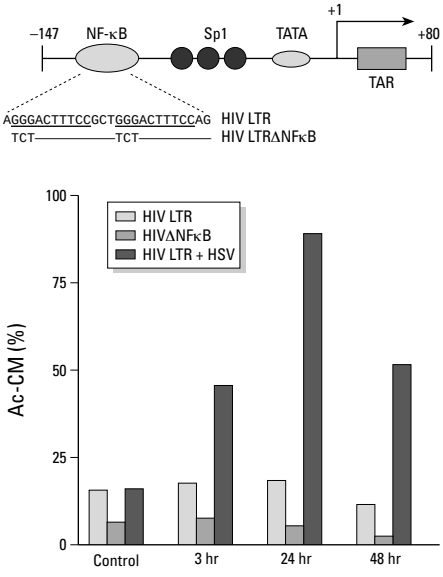

Figure 6. FB_1 treatment has no effect on NF-κB transcriptional activity. CV-1 cells were transfected with the designated constructs. Following transfection, cells were treated with FB_1 (5 μM) such that the treatment lasted until 48 hr after transfection. CAT enzymatic activity was measured as described previously (*39,52*). The amount of acetylated chloramphenicol activity (Ac-CM) was quantified in a PhosphorImager. The results are representative of three independent experiments.

Transient Activation of cdk2 by FB_1 Treatment

A previous study demonstrated that FB_1 treatment inhibited cdk2 activity in CV-1 cells (*30*). This same study suggested that 3 hr after FB_1 treatment a slight increase in cdk2 activity occurred. This may be important because cdk activity increases during the early stages of apoptosis (*32,45*). To test whether FB_1 treatment increased cdk2 levels transiently, cdk2 activity was analyzed at different times during the early stages of treatment. Relative to control cultures, cdk2 activity increased at least 2-fold between 2 and 4 hr after treatment but then rapidly declined (Figure 7). In contrast, cdk2 activity was not stimulated for 4–8 hr after CV-1 cells were plated in normal media. As expected, the amount of cdk2 activity continued to increase after serum treatment. In summary, this study demonstrated that cdk2 activity increased 2- to 3-fold at 2 and 4 hr after FB_1 treatment.

Discussion

In this study, the mechanism by which FB_1 induced apoptosis was examined in CV-1, $p53^{-/-}$ MEF, and $p53^{+/+}$ MEF. Induction of apoptosis by FB_1 does not appear to require p53 because $p53^{-/-}$ MEF undergo apoptosis following treatment (Figure 3), and the E1B 55-kDa protein did not completely inhibit apoptosis (Figures 2, 3). In $p53^{-/-}$ cells, Bcl-2 protected cells from apoptosis more efficiently (Figure 3B), suggesting that under certain circumstances Bcl-2 can interfere with

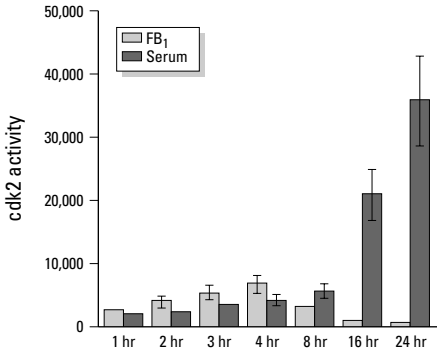

Figure 7. Measurement of cdk2 activity in CV-1 and COS-7 cells after FB_1 treatment. Total cell lysate was prepared from untreated or FB_1-treated CV-1 and COS-7 cells (5 μM of FB_1 for 3, 24, and 48 hr). Cdk2/cyclin complexes were immunoprecipitated with specific antibodies directed against the Cdk2 (Santa Cruz Biotech, Santa Cruz, CA, USA). Immunocomplexes were collected by incubation with protein A-Sepharose beads. glutathione *S*-transferase–Rb was used as a substrate to measure Cdk2 activity present in immunocomplexes as described previously (*30*). Following incubation with ^{32}P-γ-ATP, the proteins were electrophoresed on 10% SDS–PAGE, and the dried gel was autoradiographed. The results shown are the mean of four independent experiments.

FB_1-induced apoptosis. p53 transactivates expression of BAX (*46*), a pro-apoptotic gene. We hypothesize that in the absence of p53, BAX levels are lower, and under these circumstances Bcl-2 can efficiently inhibit murine fibroblasts from FB_1-induced apoptosis. It is also possible that Bcl-2 effectively inhibits apoptosis if an unknown cofactor is present.

FB_1-induced apoptosis was efficiently inhibited by CpIAP in the cell lines tested. CpIAP prevents apoptosis by interfering with the TNF/Fas pathway or binding certain caspases [reviewed in (*33*)]. Consequently, we hypothesized that the TNF/Fas pathway may be involved with FB_1-induced apoptosis. The TNFR1 recruits caspase 8, resulting in proteolytic activation of caspase 8, which subsequently activates a caspase cascade (Figure 4). The finding that caspase 8 was cleaved following FB_1 treatment (Figure 5) supports the hypothesis that the TNF/Fas pathway was activated. CpIAP also associates with the signaling molecule TNFR-associated factor (TRAF) and functions as a component of the TNFR signaling complex (*47*). TNF-α binds and activates TNFR, thus recruiting a TRAF–CpIAP complex to the cytoplasmic domain of TNFR. The TRAF–IAP complex consequently interferes with death signals activated by TNF. The recent finding that a TNFR-associated protein (TRAP)-2 is induced in CV-1 cells after FB_1 treatment lends support for the involvement of TNFR (*48*). Because CpIAP can also bind to several caspases and inhibit their protease activity (*42*), it is possible that inactivation of certain caspases by CpIAP inhibits FB_1-induced apoptosis.

Although p35 and crmA are both caspase inhibitors, p35 inhibits a broader spectrum of caspases compared to crmA (*49*). IAP family members physically interact with caspase 3, 7, and 9 [reviewed in (*33,42*); Figure 4]. crmA has been reported to bind to caspase 8, but not caspase 9. crmA partially prevented apoptosis following FB_1 treatment, suggesting that the caspase 8 pathway is not the exclusive determinant in apoptosis. Our results cannot rule out the possibility that FB_1 treatment leads to activation of caspase 9, which is a proximal caspase in mitochondria-mediated apoptosis. Unlike caspase 8, we have not been able to demonstrate that caspase 9 was cleaved following FB_1 treatment (data not shown). Because caspase 9 activation requires interaction with active Apaf-1, but not proteolytic activation (*50*), further experiments will be necessary to determine if the caspase 9 signaling pathway is also involved with FB_1-induced apoptosis.

The association of TNFR with other factors, TNFR-associated death domain, TRAF2, Fas-associated death domain, or DAXX, can lead to apoptosis or NF-κB activation (*41,51*), which prevents cell death. The finding that FB_1 treatment did not lead to NF-κB (Figure 6) or AP-1 activation (*52*) suggested that FB_1 treatment selectively activated components of the TNF pathway leading to cell death. When FB_1 is injected subcutaneously into mice (*53*), TNF-α activity increases. A TNF-α–like activity is also induced in swine after FB_1 injection (*54*). Induction of TRAP2 in CV-1 cells but not COS-7 cells (*48*) suggested that TRAP2 plays an important role in FB_1-mediated apoptosis. Taken together, we hypothesize that induction of a TNF-like pathway in FB_1-treated cells has relevance to its toxic and carcinogenic properties. Future experiments will focus on identifying the factors in the TNF pathway that are induced by FB_1.

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