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ORIGINAL PAPER

Apoptosis of Dedifferentiated Hepatoma Cells is Independent of NF-κB Activation in Response to LPS

M. Ryan Reidy · Janette Ellis · Erin A. Schmitz · David M. Kraus · Gary A. Bulla

Abstract Dedifferentiated hepatoma cells, in contrast to most other cell types including hepatoma cells, undergo apoptosis when treated with lipopolysaccharide (LPS) plus the protein synthesis inhibitor cycloheximide (CHx). We recently reported that the dedifferentiated hepatoma cells also exhibit a strong and prolonged NF-κB induction phenotype upon exposure to LPS, suggesting that NF-κB signaling may play a pro-survival role, as reported in several other cell systems. To test the role of NF-κB in preventing LPS-mediated apoptosis, we examined the dedifferentiated cell line M38. Results show that antioxidants strongly inhibited LPS + CHx-mediated cell death in the M38 cells, yet only modestly inhibited NF-κB induction. In addition, inhibition of NF-κB translocation by infection of the M38 cells with an adenoviral vector expressing an IκBα super-repressor did not result in LPS-mediated cell death. These results suggest that unlike TNFα induction, the cell survival pathway activated in response to LPS is independent of NF-κB translocation in the dedifferentiated cells. Addition of inhibitors of JNK, p38 and ERK pathways also failed to elicit LPS-mediated apoptosis similar to that observed when protein synthesis is prevented. Thus, cell survival pathways other than those involving NF-κB inducible gene expression or other well-known pathways appear to be involved in protecting the dedifferentiated hepatoma variant cells from LPS-mediated apoptosis. Importantly, this pro-apoptotic function of LPS appears to be a function of loss of hepatic gene expression, as the parental hepatoma cells resist LPS-mediated apoptosis in the presence of protein synthesis inhibitors.

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Introduction

Lipopolysaccharide (LPS), a glycolipid component of the outer wall of Gram-negative bacteria, is responsible for many of the effects noted in bacterial infections including hypotension, fever, multiple organ failure, shock, and death (Morrison and Ryan 1987). Because vascular complications of septic shock are related to endothelial cell injury (Pohlman and Harlan 1989), LPS effects on endothelial cells likely play a major role in septic shock. Numerous signaling pathways have been implicated in LPS effects, including activation of NF-κB (Muller et al. 1993; Swantek et al. 1997; Hawiger et al. 1999), and mitogen-activated protein kinase (MAPK) pathways: p42/p44/extracellular signal-regulated kinases 1 and 2 (ERK1/2) (Liu et al. 1994; Durando et al. 1998), stress-activated protein kinase/c-jun N-terminal kinase (SAPK/JNK) (Hambleton et al. 1996) and p38 MAPK (Han et al. 1994; Ono and Han 2000). However, the role of these pathways in mediating shock is unclear.

LPS has been reported to induce apoptosis of a limited number of cell types, including endothelial cells (Pohlman and Harlan 1989; Haimovitz-Friedman et al. 1997; Manna and Aggarwal 1999), macrophages (Karahashi and Amano 1998) and dedifferentiated hepatoma cells (Bulla et al. 2001). LPS-mediated apoptosis in these cell types is similar to apoptosis induced by tumor necrosis factor-α (TNFα) observed in a wide variety of cell types (Ashkenazi and Dixit 1998). Like TNFα-mediated apoptosis, LPS-mediated apoptosis normally only occurs in the absence of protein synthesis, suggesting that both TNFα and LPS activate both pro-apoptotic and anti-apoptotic pathways (Wallach 1997). Interestingly, genetic deletion of TNF receptors p60 and p80 resulted in enhanced LPS-induction of signaling pathways in mouse macrophage cells (Takada and Aggarwal 2003).

Protection of cells from TNFα-mediated cell death has been shown to be mediated through induction of NF-κB (Van Antwerp et al. 1996; Beg and Baltimore 1996). Prevention of NF-κB activation results in TNFα-mediated apoptosis even in the absence of protein synthesis inhibitors (Van Antwerp et al. 1996; Beg and Baltimore 1996; Liu et al. 1996; Wang et al. 1996). Indeed, NF-κB has been shown to transcriptionally activate expression of a number of genes that play anti-apoptotic roles including A1 (Grumont et al. 1999), Bcl-2 (Moissac et al. 1999), IEX-1 (Wu et al. 1998), and c-IAP (Liu et al. 1996). Thus, NF-κB is considered the key player in protection of cells from pro-apoptotic pathways.

In the mammalian liver, apoptosis has been observed in response to bacterial and viral infection as well as fibrosis and cholestasis, leading investigators to believe that cell death plays a causative role in each of these disease states (Patel et al. 1998). NF-κB is highly active in the regenerating liver (Taub 1996) and NF-κB (p65 subunit) knockout mice show massive liver apoptosis (Beg et al. 1995). Furthermore, NF-κB is required during liver regeneration, as inhibition of NF-κB activation resulted in striking liver apoptosis in hepatectomized rats (Iimuro et al. 1998). The effects of LPS in mice are attributed to activation of TNFα expression since TNFα-/- mice are resistant to a normally lethal dose of LPS (Marino et al. 1997).

We have utilized well-characterized dedifferentiated hepatoma variant cell lines to examine the influence of cell fate on cellular sensitivity to LPS-mediated apoptosis. Previous results suggest that regulatory factors upstream of the HNF4/HNF1α pathway are necessary for both hepatic gene expression and protecting hepatoma cells from LPS-mediated apoptosis (Kraus and Bulla 2002; Schmitz et al. 2004). We show here that

although NF-κB induction is strong in the dedifferentiated hepatoma cells, it does not appear to protect M38 cells from LPS-mediated apoptosis. Furthermore, LPS-mediated NF-κB induction in the dedifferentiated hepatoma cells appears to be by a distinct mechanism that does not require degradation of IκBα or IκBβ molecules. Last, the use of specific map kinase inhibitor molecules suggest that JNK, p38, and ERK pathways also do not provide protection from LPS mediated cell death in the dedifferentiated cells.

Materials and Methods

Cell lines and Culture Conditions

Fg-14 cells were derived from Fado-2 rat hepatoma cells as described (Bulla and Fournier 1992). Fg-14 cells were maintained in medium containing adenine-aminopterin-thymidine (AAT) to select for AT-*aprt* transgene expression. Hepatoma variant line M38 was derived from Fg-14 cells by selection in 2,6-diaminopurine (DAP) plus 6-thioxanthine (6-TX), and maintained in medium containing DAP, as described (Bulla and Fournier 1992). All cell lines were maintained in 1:1 Ham's F12:Dulbecco's modified Eagle's medium supplemented with penicillin, streptomycin, and 10% fetal bovine serum (Gibco).

Reagents

Cycloheximide (CHx), LPS (*Escherichia coli*, 055:B5), TNFα, *N*-acetylcysteine (NAC), and Pyrrolidinedithiocarbamate (PDTC) were obtained from Sigma (St. Louis, MO). Antibodies to IκBα and (κ(β were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Viability Analysis

Cells were treated with TNFα, LPS, CHx, or combinations of these compounds. At specific time points, cells were trypsinized and cell viability analysis was carried out by trypan blue exclusion (microscopic examination). Results were verified using TUNEL assays, as described (Bulla et al. 2001).

Cell Inductions and Inhibitors

Cells were treated in serum-free 1:1 Ham's F12:Dulbecco's modified Eagle's medium containing one or a combination of the following: 1 μg/ml LPS, 50 ng/ml TNFα, 10 μM CHx, 15 mM NAC, or 75 mM PDTC. Map kinase pathway inhibitors were purchased from Axxora, Inc. and included specific inhibitors to inhibit specific MAPK cascades (20 μM SP600125 for JNK; 20 μM PD98059, a MEK1/MEK2 inhibitor for ERK; and 10 μM SB202190 for p38). Fg14 and M38 cells were pretreated with cell pathway inhibitors for 1 h prior to addition of LPS.

Adenoviral Infections

The adenoviral vectors containing the IκBα mutant (S32A/S36A) cDNA and lacZ cDNA were kindly provided by R. Rippe (University of North Carolina). Large batches

of adenovirus were prepared by infecting 293 cells in spinner cultures. Infected cells were lysed and the adenovirus purified on two consecutive cesium chloride centrifugation gradients. The purified virus was stored at –80°C in 10% glycerol, 10 mM Tris–HCl, pH 7.4, and 1 mM $MgCl_2$. Viral stocks were determined to be at a concentration of approximately 2×10^{11} PFU/ml. Dedifferentiated M38 cells were infected at a multiplicity of infection (MOI) of 10–40/cell in serum free medium. After 1 h, the virus mixture was replaced with complete medium. Approximately 3 days post infection, cells were treated with LPS, CHx, or LPS + CHx and assayed for NF-κB at 5 h or apoptosis at 14 h.

Nuclear Extracts

Nuclear extracts were prepared according to the method of Schreiber et al. (Schreiber et al. 1989) with modifications. Briefly, cells were removed with a cell scraper, centrifuged for 3 min at $550 \times g$, supernatant was discarded, and the pellet was resuspended in 400 μl of chilled buffer I (5 mM $MgCl_2$, 5 mM β-glycerol phosphate, 0.2 mM EDTA (or EGTA), 0.3 M sucrose, 1 mM DTT, 0.5 mM PMSF, 1X protease inhibitor cocktail, 20 mM Tris–HCl, pH 7.8). The protease inhibitor cocktail was 6.0 ng/ml leupeptin, 0.1 μg/ml aprotinin, 40 μM benzamidine, and 20 ng/ml antipain. NP-40 was added to a concentration of 0.5% and the samples were incubated on ice for 1 min before being spun for 3 min at $2,200 \times g$. The supernatant was discarded and the pellet was resuspended in 100 μl of chilled buffer II (5 mM $MgCl_2$, 350 mM NaCl, 0.2 mM EDTA, 10 mM β-glycerol phosphate, 25% glycerol, 0.5 mM DTT, 0.5 mM PMSF, protease inhibitor cocktail, 10 mM Tris–HCl, pH 7.8). Samples were incubated on ice for 15 min and centrifuged for 15 min at 4°C at $10,000 \times g$. The pellet was discarded and the supernatant was transferred to a new tube and stored at –70°C.

Electrophoretic Mobility Shift Assay (EMSA)

About 10 μg of nuclear protein were added to 4 mM HEPES, 10 mM NaCl, 0.3 mM $MgCl_2$, 2% glycerol, 0.6–1.2 μg of poly-dIdC, and a 1X protease inhibitor cocktail described in the nuclear extract procedure. To this solution, 1×10^4 cpm of the labeled oligonucleotide was added and incubated with the protein for 15–30 min at 4°C. The NF-κB oligonucleotide contained the sequence AGTTGAGGGGACTTTCCCAGGC, and the Oct-1 oligonucleotide contained the sequence GGGGGTAATTTGCATTTC-TAAGGG. Dye was added and the samples were subjected to polyacrylamide gel electrophoresis for 2–3 h at 7.5 V/cm in a non-denaturing polyacrylamide gel with 1X TBE buffer (0.45 M Tris base, 0.44 M boric acid, and 0.01 M EDTA, pH 8.0). The gel was then placed on Whatman filter paper, dried, and exposed to film for 1–5 days.

Western Blot Analysis

About 10 μg of protein was boiled at 100°C for 10 min in 1X SDS gel-loading buffer (2% sodium dodecyl sulfate SDS, 0.1% bromophenol blue, 10% glycerol, 100 mM dithiothreitol, 50 mM Tris–HCl, pH 6.8). Samples were then loaded onto a denaturing polyacrylamide gel. The denatured protein was electrophoresed for 1.5 h at 100 volts. The protein was then transferred to an Immobilon™-P PVDF membrane (MILLI-PORE) and incubated overnight at 4°C in blocking solution containing Tris Buffered

Saline (TBS) plus 5% non-fat dried milk and 0.05% Tween-20. TBS is 20 mM Tris base, 137 mM NaCl, 3.8 mM HCl and a final pH of 7.6. The membrane was probed for 1 h with the primary antibody diluted 1:750 in blocking solution and then the secondary antibody diluted 1:500 in blocking solution.

The blot was then visualized according to the specifications of ECL^{TM} 2209 Western Blotting Detection Kit (Amersham Life Sciences). This kit uses the chemiluminescent oxidation of luminol by horseradish peroxidase (HRP) and hydrogen peroxide. Thus, secondary antibodies were conjugated to HRP. Briefly, detection solution 1 was mixed with an equal volume of detection solution 2 and then added to the protein side of the membrane for 1 min. After the detection solution was drained, the membranes were exposed to film for 30 s to 1 h.

Results

Both CHx and LPS Induce NF-κB without Inducing Apoptosis

We have previously shown that HNF4$^-$/HNF1α^- dedifferentiated hepatoma cells undergo apoptosis upon exposure to LPS plus CHx. These cells were derived from Fado-2 hepatoma cells (see Fig. 1A) using a positive/negative selection scheme (Bulla 1997). In contrast, the parental hepatoma cells as well as other hepatoma cell lines are resistant to the exposure of LPS + CHx. Restoration of hepatic function by the
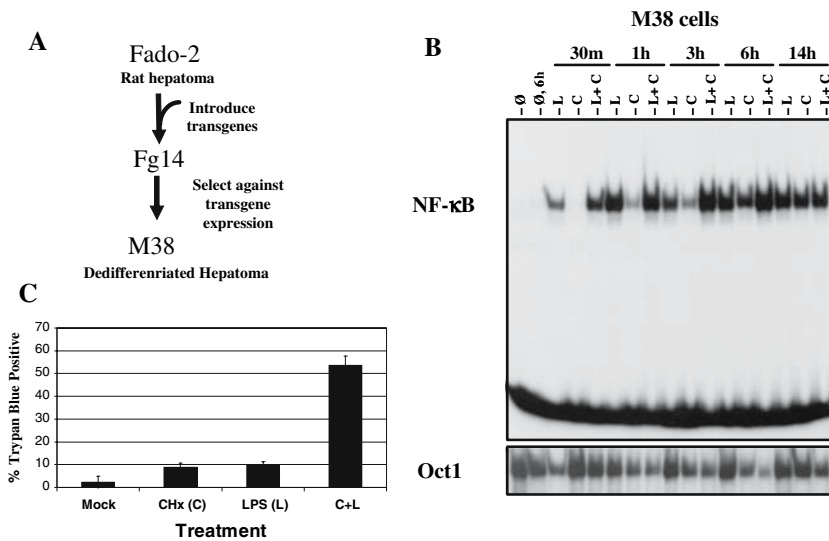
Fig. 1 Cycloheximide and LPS induce NF-κB additively. (**A**) M38 cells were derived from Fg14 hepatoma cells by selection against introduced liver-specific transgenes. (**B**) M38 cells were exposed to LPS (1 μg/ml), CHx (10 mM), or LPS + CHx for 30 min, or 1, 3, 6, and 14 h. Nuclei were isolated, lysed, and nuclear extracts prepared. About 10 μg of each extract was incubated with an NF-κB oligonucleotide and complexes were resolved on a 4% non-denaturing polyacrylamide gel. Oct1 (lower panel) levels were monitored to control for nuclear extract quality. (**C**) M38 cells were exposed to the indicated compounds for 24 h and then cell death monitored using trypan blue exclusion. Results shown are averages and standard deviation values from triplicate experiments. M38 = dedifferentiated cells; Mock = untreated cells; L = lipopolysaccharide; C = cycloheximide

introduction of HNF4 and/or HNF1α into the dedifferentiated cells rendered them resistant to cell death (Bulla et al. 2001). We believe these results suggest that LPS activates both cell death and cell survival pathways in the dedifferentiated hepatoma cells and that the prevention of protein synthesis using CHx allows the pro-death pathways to dominate. Because of the known role of NF-κB in preventing TNFα-mediated apoptosis, we asked whether NF-κB induction was responsible for the failure of LPS alone to induce apoptosis of the dedifferentiated cells in the absence of CHx.

First, we examined the time course of NF-κB induction by LPS, CHx, and LPS + CHx in the M38 cells (see Fig. 1B). LPS induced NF-κB translocation within 30 min, a response which appeared to reach a maximum at 1 h. This induction remained strong through the 14 h time point. In contrast, CHx treatment resulted in no detectable signal until 1 h, and then produced a weak signal at 1 and 3 h time points. However, by 6 h, NF-κB induction became strong. The combination of LPS + CHx treatment resulted in a robust signal at 30 min, which remained strong through the 14 h time point. We also examined the level of apoptosis occurring in the drug-treated M38 cells using trypan blue exclusion a technique, which we have found to provide high reliability with other measures of apoptosis (Bulla et al. 2001). In agreement with previous results (Kraus and Bulla 2002), the combination of LPS + CHx resulted in greater than 50% cell death, whereas the drugs individually produced less than 10% cell death (Fig. 1C). Thus, the combined LPS + CHx induction results in a rapid, strong and long-lasting NF-κB induction phenotype accompanied by a high degree of cell death.

We next asked whether antioxidants affected M38 cell survival. Consistent with previous data (Bulla et al. 2001), NF-κB induction levels in the M38 cells were much higher than those in the parental Fg14 cell line in response to LPS or LPS + CHx (Fig. 2A). We therefore asked whether NF-κB induction was inhibited by pre-incubation of the cells with NAC or PDTC prior to treatment with LPS + CHx. At 10 h post-induction, NF-κB induction levels were reduced although not ablated in the M38 cells by pre-incubation with NAC or PDTC (Fig. 2A). The Fg14 cells also showed a decreased induction level, although the signal was weak initially. Cell death assays showed that both NAC and PDTC significantly reduced LPS + CHx cell death in the M38, reducing death from 70% to 10% and 25%, respectively (Fig 2B). No effect of these compounds was observed using the parental Fg-14 cell line (Fig. 2B).

LPS does not Induce Cell Death when NF-κB Translocation is Prevented by an IκBα Super-repressor

The above results suggest that NF-κB induction is not required to protect the M38 cells from LPS-mediated apoptosis. To confirm that LPS-mediated apoptosis is independent of NF-κB induction we used adenoviral-mediated gene transfer. M38 cells were infected with adenoviral constructs encoding either β-galactosidase or a IκBα super-repressor (IκBdm), containing mutations at serines 32 and 36, thereby preventing phosphorylation of IκB and dissociation from NF-κB. Infected cells were then treated with CHx, LPS or CHx + LPS and harvested at 5 h post-treatment.

Using a multiplicity of infection of 250 infectious viral particles/cell, we achieved 100% infection frequency with the Ad-lacZ construct (data not shown). As previously observed (Fig. 1A), results show that NF-κB is strongly induced in the uninfected cells upon exposure to CHx, LPS, or LPS + CHx (Fig. 3A). However, Ad-IκBdm infection resulted in complete suppression of NF-κB induction in each case (Fig. 3A). Expression of the mutant and wild-type IκBα was readily detected in the cell extracts (Fig 3B). The
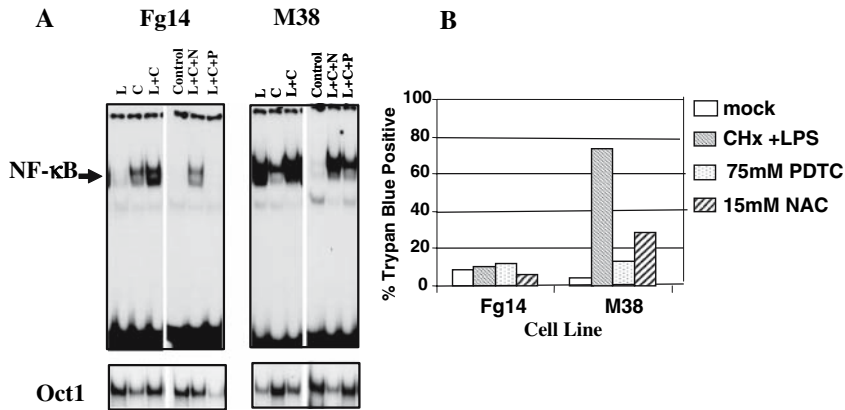
Fig. 2 Both cycloheximide and LPS induce NF-κB. (**A**) Fg14 and M38 cells were exposed to LPS (1 μg/ml), CHx (10 μM), or LPS + CHx in the presence or absence of 75 mM PDTC or 15 mM NAC for 10 h. Nuclei were isolated, lysed, and nuclear extracts prepared. A total of 10 μg of each extract was incubated with an NF-κB oligonucleotide and complexes were resolved on a 4% non-denaturing polyacrylamide gel. Oct1 (lower panels) levels were monitored to control for nuclear extract quality. (**B**) Fg14 and M38 cells were exposed to LPS (1 μg/ml), CHx (10 μM), or LPS + CHx in the presence or absence of 75 mM PDTC or 15 mM NAC for 24 h. Apoptosis was scored using trypan blue exclusion. Results shown are averages and standard deviation values from triplicate experiments. Fg14 = parental hepatoma cells; M38 = dedifferentiated cells; L = lipopolysaccharide; C = cycloheximide; N = N-acetyl cysteine; P = PDTC

mutated IκBα migrated slower than the endogenous IκBα. Notably, infection with the Ad-IκBαdm construct at 250 MOI did not reduce cell death in LPS, CHx, or LPS + CHx treated cells compared to non-infected controls or the LacZ-infected controls (Fig. 3C). These results further argue that NF-κB induction is not required to protect the dedifferentiated cells from apoptotic mechanisms induced by LPS.

LPS + CHx, but Neither Alone, Results in Degradation of IκBα and IκBβ

LPS-stimulation of pre-B cells results in degradation of both IκBα and IκBβ (Thompson et al. 1995), whereas endothelial cells degrade only IκBα (Zen et al. 1999). We asked if these molecules were degraded in the M38 cells in response to LPS, CHx, or LPS + CHx. Results show that neither LPS or CHx alone results in degradation of either IκBα or IκBβ. However, the combination of both LPS + CHx resulted in degradation of both of these molecules (Fig. 4A) in 60 min or 4 h, respectively. In contrast, the parental Fado-2 hepatoma cells (Fado-2 cells are the parental cell line of the Fg-14 cells) showed a transient loss of both IκBα and IκBβ (in response to CHx alone, followed by re-expression of these molecules. As with the M38 cells, both molecules were degraded upon treatment of the Fado-2 cells with LPS + CHx (Fig 4B).

Other Pathway Inhibitors do not Enhance LPS-mediated Cell Death

Because the most likely pathway, NF-κB, does not appear to be responsible for protecting M38 cells from LPS-mediated cell death, we asked about the involvement of other signaling pathways. Three pathways known to be activated by LPS include the JNK, ERK, and p38 pathways, all of which are MAP kinase pathways activated in
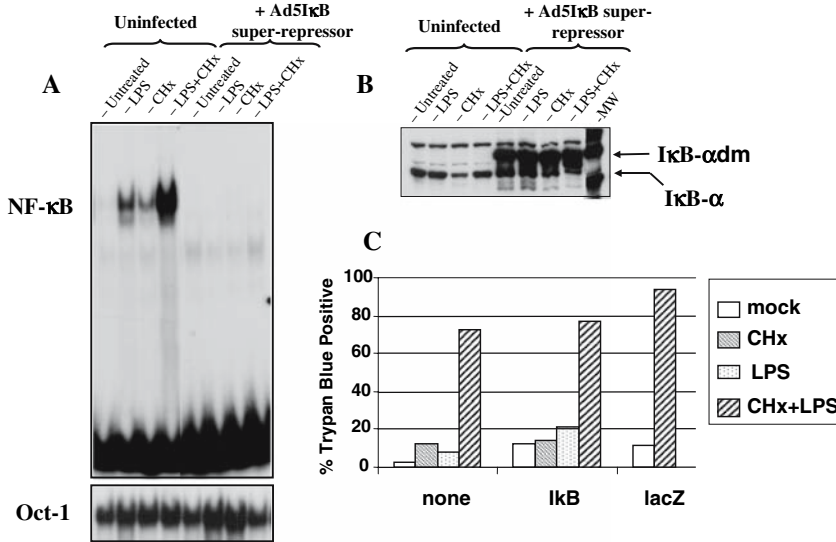
Fig. 3 NF-κB inhibition does not result in LPS-mediated cell death. M38 cells were mock-infected or infected with adenoviral constructs Ad-LacZ or Ad-IκBαdm at a multiplicity of infection of 250 pfu/cell. At 24 h post-infection, cells were treated with LPS (1 μg/ml), CHx (10 μM), or LPS + CHx for 24 h. Nuclear extracts were prepared and assayed for (**A**) NF-κB by EMSA (using Oct1 binding as a control) and (**B**) IκBα expression by Western analysis. (**C**) M38 cells treated as describe above were scored for apoptosis using trypan blue exclusion. As a control for infection efficiency, cells were infected with an AD-lacZ virus. Nearly 100% of cells stained blue (results not shown). LPS = lipopolysaccharide; CHx = cycloheximide
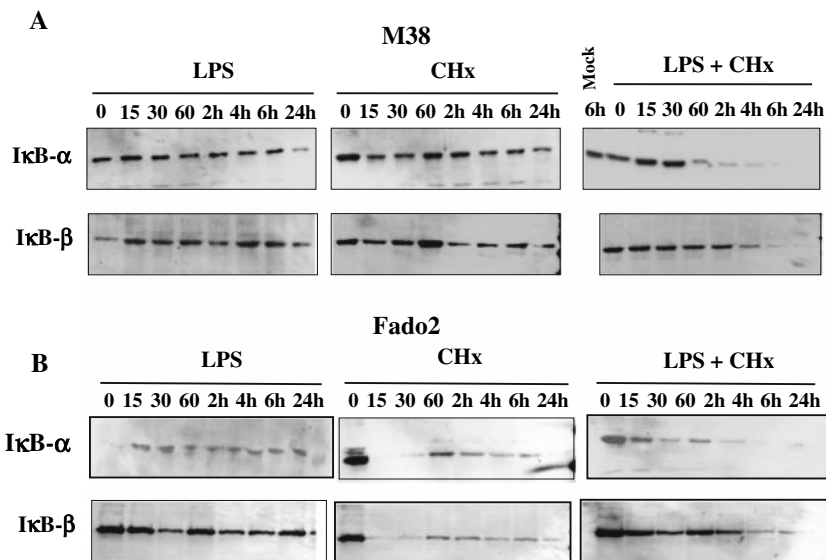
Fig. 4 LPS and CHx-mediated NF-κB induction is independent of IκBα and IκBβ degradation in M38 cells. M38 cells were treated with LPS (1 μg/ml), CHx (10 μM), or LPS + CHx for 0, 15, 30, 60 min, and 2 4, 6, and 24 h. Cells were harvested for western analysis of IκBα and IκBβ levels. LPS = lipopolysaccharide; CHx = cycloheximide
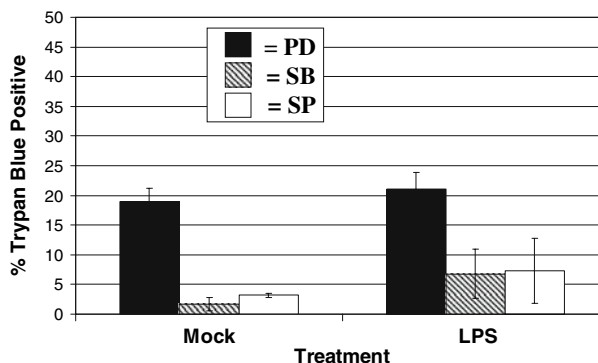
Fig. 5 JNK, p38, and ERK inhibitors do not result in LPS-mediated cell death. M38 cells were pre-incubated for 30 min with inhibitors to inhibit specific MAPK cascades (20 μM SP600125 for JNK; 20 μM PD98059, a MEK1/MEK2 inhibitor for ERK; and 10 μM SB202190 for p38) Cells were then exposed to LPS (1 μg/ml) for 24 h and cell death monitored using trypan blue exclusion. Results shown are averages and standard deviation values from at least three independent assays. Standard error bars are shown. LPS = lipopolysaccharide; CHx = cycloheximide

several cellular responses (Liu et al. 1994; Durando et al. 1998; Hambleton et al. 1996; Han et al. 1994; Ono and Han 2000). Pretreatment of M38 cells with the ERK and p38 pathway inhibitors (PD98059 and SB202190, respectively) in the presence of LPS did not result in substantially increased cell death. Pretreatment with the JNK pathway inhibitor SP600125 resulted in increased cell death, but was independent of the presence of LPS (Fig. 5). Thus, of the pathways known to be induced by LPS, none of them appear to be responsible for the dramatic levels of apoptosis observed due to inhibition of protein synthesis in the presence of LPS.

Discussion

The dedifferentiated hepatoma cells serve as a model to understand the influence of liver-specific gene expression on cellular response pathways. We previously reported that the dedifferentiated hepatoma cell lines (but not human, rat or mouse hepatoma cell lines) undergo apoptosis in response to treatment with LPS + CHx (Bulla et al. 2001). In addition, these cells showed an unusually strong NF-κB induction phenotype (Bulla et al. 2001). Contrary to expected results, the degree of NF-κB induction is not required to protect cells from LPS-mediated apoptosis in the absence of CHx. Because NF-κB has been shown to protect several cell types, including hepatic cells, from undergoing apoptosis in response to TNFα (Van Antwerp et al. 1996; Beg and Baltimore 1996; Wang et al. 1996), we further characterized the dedifferentiated cells to understand the LPS-induction phenotype.

Both CHx and LPS were able to induce NF-κB translocation in both the M38 cells and the hepatoma parental cell (Fado-2), although the M38 cells consistently show a much stronger induction. Also, the synergistic effect of CHX and LPS on NF-κB induction suggests that distinct mechanisms are used to direct NF-κB translocation. Interestingly, although two antioxidants, NAC and PDTC, significantly reduced cell death in cells treated with LPS and CHx, neither completely reduced NF-κB induction.

Similar results were reported for other cell types, including pre-B cells, mouse fibroblasts, and Jurkat cells (reviewed in Schreck et al. (1992)). These results suggest that cell death is affected by the oxidation state of the cells, but the protective effect is not through modulation of NF-κB translocation. The effect of reactive oxygen intermediates may play a key role in apoptosis of these cells, a possibility which has not yet been tested.

Most reports indicate that NF-κB activation protects cells from TNF-induced apoptosis (Manna and Aggarwal 1999; Van Antwerp et al. 1996; Beg and Baltimore 1996; Liu et al. 1996; Wang et al. 1996; Chu et al. 1997). However, other reports suggest that NF-κB is not involved in the prevention of TNFα-induced apoptosis (Cai et al. 1997; Guo et al. 1998). Indeed, over expression of IκBα or a dominant negative form of IκBα in MCF7 breast carcinoma cells prevented NF-κB induction, yet failed to alter TNFα-mediated cell death (Liu et al. 1996; Cai et al. 1997). These differences are likely attributable to different cell lines used. In this study, we found that LPS-induced apoptosis of dedifferentiated hepatoma cells was independent of NF-κB activation.

Our studies also found that NF-κB signaling in the dedifferentiated cells is distinct from other cell types. Zen et al. (1999) reported that LPS-induced activation of NF-κB in human endothelial cells involved transient degradation of IκBα but not IκBβ. Thompson et al. (1995) reported that 70Z/3 pre-B cells treated with LPS resulted in a transient degradation of both IκBα and IκBβ. Our results show neither of these molecules are degraded in the M38 cells, even transiently, with either LPS or CHx alone. However, we cannot rule out short-term loss of these molecules, as we did not examine time points prior to 15 min post-induction. However, the data suggests that only when protein synthesis is inhibited do these molecules become degraded. Thus, it is unlikely that IκBα or IκBβ molecules are involved in NF-κB induction observed in response to LPS. These results suggest a mechanism for induction of NF-κB that is independent of phosphorylation at serines 32 and 36 of IκBα. This observation merits further investigation because it implies a novel mechanism of NF-κB induction, perhaps through a pathway which phosphorylates IκBα at other peptide residues. Our attempts to identify other known LPS-induced pathways (JNK, p38, and ERK) that could be responsible for pro-survival signaling were not fruitful. Thus, CHx must prevent synthesis of proteins required for cell survival in response to LPS, but is independent of known pathways. The fact that CHx alone only accounts for a low degree of cell death makes it unlikely that simple lack of protein synthesis is causing cell death in the absence of LPS.

Conclusions

Our results suggest that, unlike other liver-derived cells tested, LPS exposure of the dedifferentiated hepatoma cells results in activation of both pro-survival and pro-death pathways, but that the pro-survival response is independent of NF-κB induction. Thus, one or more additional pathways must be activated to prevent LPS-mediated cell death in the M38 cells, although drugs used to prevent activation of ERK, JNK, and p38 pathways failed to show a role in these well-known map kinase pathways in this role. Because hepatoma cells do not undergo apoptosis in response to LPS + CHx (Kraus and Bulla 2002), the unusual phenotype of the dedifferentiated cells is likely due to the absence of loci directing hepatic gene expression. Understanding the relationship

between tissue-specific gene expression and response to LPS may provide insight into the role of tissue-specific transcription factors in cellular response mechanisms.

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