Nuclear Factor-κB/Rel Blocks Transforming Growth Factor β1-induced Apoptosis of Murine Hepatocyte Cell Lines

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Abstract

TGF-β1-induced apoptosis is a major mechanism for the carcinogenic effects of tumorigenesis. Recently, NF-κB has been implicated in the regulation of cell survival. We show here that NF-κB is required for the survival of murine hepatocytes. Treatment of hepatocytes with transforming growth factor β1 (TGF-β1) induces growth arrest, which is followed by extensive cell death by apoptosis. Furthermore, we found that TGF-β1 down-regulates NF-κB activity in murine B cell lymphomas, inducing apoptosis. Additionally, NF-κB-deficient mice died during gestation due to apoptosis of liver cells. Here we have explored the effects of TGF-β1 on hepatocytes, using two untransformed murine hepatocyte cell lines, AML-12 and NMH, which constitutively express classical NF-κB. TGF-β1 treatment caused increased NF-κB binding that was followed by a dramatic decrease in NF-κB levels that preceded apoptosis. Ectopic c-Rel expression ablated apoptosis induced by TGF-β1. The down-regulation in NF-κB activity correlated with elevated IκB-α expression due to hypophosphorylation and increased IκB-α protein stability. Thus, NF-κB factor expression acts directly to promote liver cell survival. Furthermore, these findings characterize a novel signaling pathway for TGF-β1 in epithelial cells involving down-regulation of NF-κB/Rel factors activity through posttranslational modification of IκB-α protein.

Introduction

NFκB was originally defined as the NF that bound to the B site of the κ light chain gene enhancer in B lymphocytes (1). NFκB is now known to be a family of dimeric transcription factors, with subunits that contain an NH2-terminal domain of approximately 300 amino acids that shares homology with the v-rel oncogene (2). Classical NFκB is composed of a p50 (NFκB1) and p65 (RelA) subunit and binds very avidly to κB elements (2). In addition, other Rel-related subunits have been identified, including c-Rel, RelB, and p52 (NFκB2; Ref. 3). NFκB was found to be ubiquitously expressed in non-B cells, sequestered in the cytoplasm with specific inhibitory proteins termed IκBs. Activation and nuclear localization can be induced in these cells by a variety of agents (4, 5). Following stimulation, IκB protein was found to be phosphorylated on two conserved serine residues, leading to degradation via the proteasome pathway. The released NFκB/Rel dimers are then free to migrate to the nucleus (2, 6, 7).

NFκB factors control expression of genes mediating immune and inflammatory responses, adhesion molecules, and growth (reviewed in Refs. 2–4). Previously, v-rel had been implicated in avian B lymphocyte survival (8, 9). Recently, cellular Rel factors have been shown to play a role in apoptosis. We demonstrated that inhibition of constitutive NFκB/Rel activity in murine B-cell lymphomas induces cell death, and that ectopic c-Rel expression provides survival signals (10). The finding that NFκB is a survival factor has been extended by other groups to different cell types that do not display constitutively active NFκB, including fibroblasts, macrophages, and epithelial and T-cell lines (11–14).

Hepatocytes are the major cellular constituent of the liver. These epithelial cells are normally quiescent, but following two-thirds PH, they rapidly and synchronously re-enter the cell cycle until the liver regains its original mass (15). PH induces expression of classical NFκB in hepatocytes (16–18), and two untransformed and well differentiated murine hepatocyte cell lines, termed AML-12 and NMH (19), express classical NFκB constitutively (17). TGF-β1 belongs to the TGF superfamily of cytokines that have been implicated in the regulation of growth, differentiation, development, and more recently, apoptosis (20–22). TGF-β1 treatment of cultured hepatocytes inhibits DNA synthesis (23–25) and leads to extensive cell death by apoptosis within 24 h (26). In addition, hepatic expression of mature TGF-β1 in transgenic mice results in hepatic fibrosis and apoptotic cell death of hepatocytes (27). Furthermore, TGF-β1 inhibits hepatocyte cell proliferation in vivo after PH (28). Recently, we demonstrated that the TGF-β1-mediated induction of apoptosis of WEHI 231 and CH33 B-cell lymphomas resulted from NFκB/Rel inactivation (29). Interestingly, mice deficient in the p65 subunit of NFκB were found to display embryonic lethality within 14 days of gestation, resulting from massive liver degeneration due to apoptosis of hepatocytes (30). Although it was unclear whether this was a direct effect, together the above results suggested a role for NFκB-related factors in hepatocyte survival. Thus, we

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3 The abbreviations used are: NF, nuclear factor; PH, partial hepatectomy; TGF-β1, transforming growth factor β1; URE, upstream response element; GST, glutathione S-transferase; CAT, chicken phospho thymidine kinase; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy méthoxyphenyl)-2-(4-sulfophenyl)-4H-tetrazolium inner salt; EMSA, electrophoretic mobility shift assay.
tested the role of NF-κB/Rel factors in TGF-β1-mediated apoptosis of hepatocytes using AML-12 and NMH cells. TGF-β1 treatment caused extensive cellular death via apoptosis that was preceded by a decrease below basal levels in NF-κB binding due to decreased phosphorylation and resultant stabilization of IκB-α protein. Ectopic c-Rel expression significantly reduced the extent of growth inhibition and apoptosis induced by TGF-β1. These results extend the role for NF-κB/Rel factors as survival genes to hepatocytes and dissect a novel TGF-β1 signaling pathway through IκB-α hypophosphorylation and NF-κB/Rel factors in these epithelial cells.

Results

AML-12 Cells Express Constitutive NF-κB Activity. We first sought to characterize NF-κB/Rel-related factors binding activity to κB elements in AML-12 cells. Mobility shift analysis was performed with the upstream (URE)-κB element from the c-myc gene as probe (31). Nuclear extracts from exponentially growing AML-12 cells displayed two constitutive binding complexes with the URE, termed bands 1 and 2 (Fig. 1A, Lane 1), not seen with probe alone (Fig. 1A, Lane 9). Competition with 100× molar excess of cold URE probe confirmed the specificity of this binding (Fig. 1A, Lane 8). To identify the nature of the NF-κB/Rel subunits, antibody preparations against RelA (p65), NF-κB1 (p50), and c-Rel subunits were used, as well as bacterially expressed IκB-α protein. Addition of an antibody against the p65 subunit resulted in complete supershift of band 1 while causing only a slight alteration in the mobility of band 2 (Fig. 1A, Lane 2). Incubation of the same antibody with the radiolabeled URE probe in the absence of nuclear lysate confirmed the specificity of the supershifted complex (Fig. 1A, Lane 3). Addition of an antibody that preferentially recognizes p50 homodimers resulted in complete supershift of band 2 (Fig. 1A, Lane 4). This supershift could be prevented by co-incubation of the anti-p50 antibody with the cognate peptide, thus indicating the specificity of the reaction (Fig. 1A, Lane 5). Furthermore, three different antibody preparations against c-Rel failed to shift or significantly reduce either band (Fig. 1A, Lane 6 and data not shown). Finally, addition of an IκB-α-GST protein blocked completely formation of band 1 without affecting the intensity of band 2 (Fig. 1A, Lane 7). Thus, we conclude that band 1 represents binding of classical NF-κB (p50/p65 heterodimers), and band 2 represents binding of p50 homodimers, consistent with the findings of Fitzgerald et al. (17). Similar results were obtained when nuclear lysates of NMH cells were subjected to bandshift analysis using the URE probe (Ref. 32 and data not shown). To test for functional NF-κB activity, AML-12 cells were transfected with a CAT reporter vector in which the TK heterologous promoter is driven by two copies of the URE element (EB; Refs. 31 and 33). As control, a similar construct (dmE8) containing two copies of a mutant version of the URE element with two G-to-C conversions, which is unresponsive to NF-κB transactivation (31), was used. The basal activity of the E8 vector in exponentially growing AML-12 cells was much greater (approximately 19-fold) than the dmE8 construct (Fig. 1D). Similar results were obtained when the same constructs were transfected in the NMH cell line (data not shown). Thus, the constitutive classical NF-κB in these untransformed murine hepatocyte cell lines is functionally active.

TGF-β1 Down-Modulates Constitutive NF-κB Activity of AML-12 Cells. TGF-β1 has been found to promote inhibition of cell proliferation and apoptosis in primary cultured hepatocytes (26). To test the effects of TGF-β1 on AML-12 and NMH cells, cultures were incubated in medium containing TGF-β1 or BSA-carrier solution as control for 6, 12, 24, or 48 h. Cell proliferation and cell viability were monitored by conversion of MTS to its formazan and by trypan blue staining, respectively. A significant decrease in both cell proliferation (Fig. 2A) and viability (Fig. 2, B and C) was noted by 24 h of TGF-β1 treatment of either cell line, which was even more pronounced by 48 h. Furthermore, propidium iodide-stained cells displayed several hallmarks of apoptosis, including chromatin condensation, pyknotic nuclei, and membrane blebbing (data not shown; see Fig. 7, AML-N1 cells, AML cells stably transfected with a pSV2Neo control vector DNA, and cells treated with TGF-β1). Thus, TGF-β1 treatment causes apoptosis of AML-12 and NMH murine hepatocytes.

Recently, we have shown that TGF-β1 inhibits NF-κB/Rel factor activity in murine B-cell lymphomas (29). To determine whether TGF-β1 would similarly down-modulate NF-κB activity in murine hepatocytes, EMSA was performed using nuclear extracts from AML-12 and NMH cells treated with TGF-β1 or BSA-carrier solution (Fig. 1, B and C). Treatment of either cell line with TGF-β1 caused a transient induction in NF-κB binding at the 3-h time point (better displayed in Fig. 4) that was followed by a significant decrease in binding levels after 6 h; furthermore, binding was well below baseline levels at the 10-h time. In contrast, the binding of the octamer transcription factor, Oct-1, was only slightly affected by TGF-β1 treatment (Fig. 1, B and C), indicating the specificity of the inhibition of NF-κB binding by TGF-β1. The effects of TGF-β1 treatment on NF-κB transcriptional activity were assessed next. AML-12 cells were pretreated for 12 h with TGF-β1 or BSA-carrier solution and transfected by electroporation with the E8 and dmE8 reporter constructs. Following incubation for an additional 8 h, cells were harvested, and CAT activity was monitored. TGF-β1 treatment reduced E8 construct CAT activity approximately 3-fold, whereas dmE8 CAT activity was essentially unaffected (Fig. 1D). The luciferase activity of a vector driven by the SV40 early promoter (pGL2-SV40), which displays high constitutive activity in AML-12 cells, was only modestly affected (Fig. 1D). Taken together, these data demonstrate that TGF-β1 treatment selectively inhibits NF-κB activity in murine hepatocyte cell lines. In addition, TGF-β1-mediated down-regulation of NF-κB activity occurs before a significant loss of cell viability.

Ectopic Expression of c-Rel Prevents TGF-β1-mediated Down-Regulation of NF-κB/Rel Activity. Because the drop in NF-κB activity correlated with TGF-β1-mediated apoptosis, we sought to determine whether constitutive expression of a transactivating subunit, such as c-Rel, could prevent cell death. AML-12 cells were stably transfected with either the neomycin resistance construct pSV2Neo or pSV2Neo plus a murine c-Rel expression vector (33). Clonal transfectants were assayed by immunoblotting for c-Rel ex-
Expression using total cellular protein isolated from cells in exponential growth. Parental AML-12 cells and all of the pSV2Neo clones isolated, as exemplified by clone AML-N1, express only extremely low levels of c-Rel protein (Fig. 3 and data not shown). This finding is in agreement with our inability to detect c-Rel binding activity in the less sensitive supershift EMSA, described above. In contrast, two of the c-Rel transfected clones, AML-R2 and R5, expressed significantly higher levels of c-Rel protein. Specificity of the anti-Rel antibody was confirmed by competition with the cognate peptide (Fig. 3). Equal loading of the samples was assessed by hybridization of the same filters with an antibody against IκB-α (Fig. 3). Expression of c-Rel in the AML-R2 cells was also confirmed by immunofluorescence and Northern blot analyses (data not shown).

The c-Rel-expressing AML-12 clones were next characterized for NF-κB DNA binding activity. A significant increase in binding to the URE probe was observed in the nuclear lysate from exponentially growing AML-R2 and AML-R5 cells when compared to that of the AML-N1 clone (Fig. 4 and data not shown).
Fig. 3. Ectopic c-Rel expression in AML-12 cell clones. AML-12 cells were transfected with 2 μg of pSV2Neo and 38 μg of murine c-Rel expression vector (AML-R2 and AML-R5) or the pSV2Neo vector alone (AML-N1) and stable cell lines selected for G418 resistance. Total cellular extracts were isolated from AML-12 (AML), AML-N1 (N1), AML-R2 (R2), and AML-R5 (R5) cells in exponential growth, and samples (40 μg) were subjected to immunoblot analysis, using an antibody preparation against the c-Rel subunit (SC-070) in the absence (upper panel) or in the presence (lower panel) of the cognate peptide. As control for equal loading, the same filters were hybridized with an antibody specific for IκB-α protein (SC-371). Position of molecular mass markers are given (kD).

not shown). TGF-β1 treatment caused a similar early transient induction of NF-κB binding activity (approximately 2-fold) at the 3-h time point in both the AML-N1 and AML-R2 clones (Fig. 4A). However, in contrast to the 9-fold decrease normally seen by 24 h in AML-12 or AML-N1, as judged by densitometric scanning (Figs. 1B and 4A), the AML-R2 cells maintained significantly higher levels of binding activity (60% relative to untreated cells). Similarly, higher levels of NF-κB binding activity were retained by AML-R5 cells compared to the AML-N1 cells following 24 h of TGF-β1 treatment (87% versus 17%; Fig. 4B). No significant changes were detected in binding to an Oct-1 probe, demonstrating equal loading of proteins and specificity of TGF-β1 effects. Thus, ectopic c-Rel expression led to maintenance of elevated levels of binding activity.

The retained NF-κB in the AML-R2 and AML-R5 clones was transcriptionally functional following TGF-β1 treatment. AML-N1, AML-R2, and AML-R5 were pretreated for 12 h with TGF-β1 or BSA-carrier solution and then transfected with E8-CAT DNA, as above. TGF-β1 treatment of AML-N1 cells repressed E8-CAT activity by approximately 3-fold (Fig. 5), in good agreement with the inhibition observed above for the wild-type AML-12 cells (Fig. 1D). The basal level of CAT activity in AML-R2 and AML-R5 cells was higher than that in AML-N1 cells (Fig. 5), consistent with the differences noted in basal NF-κB/Rel binding activity among these clones (Fig. 4). Furthermore, unlike its effect in AML-12 or AML-N1, TGF-β1 treatment of AML-R2 and AML-R5 cells yielded only modest effects on CAT activity (Fig. 5). Thus, ectopic expression of c-Rel leads to maintenance of NF-κB/Rel transcriptional activity upon TGF-β1 treatment.

assessed by conversion of MTS dye to its formazan product. Data are plotted as the percentage of converted formazan by TGF-β1-treated cells relative to that of control cells. B and C, exponentially growing AML-12 (B) and NMH (C) cells were treated, in duplicate, as above, and cell viability was determined by trypan blue exclusion assay. Values are plotted as the percentage of viable TGF-β1-treated cells relative to BSA-carrier solution-treated cells. SD (bars) was obtained using Student’s t test.
Fig. 4. Ectopic expression of c-Rel leads to maintenance of NF-κB binding activity upon TGF-β1 treatment. A, nuclear extracts were isolated from AML-N1 (N1) and AML-R2 (R2) in exponential growth (E) or following treatment with 2 ng/ml TGF-β1 for 3, 6, 10, and 24 h. EMSA was performed as described in the legend of Fig. 1. B, extracts were similarly prepared from exponentially growing AML-N1 and AML-R5 (N1 and R5, respectively) or after 24 h of treatment with TGF-β1 (T) or BSA-carrier solution (B) and subjected to EMSA for NF-κB or Oct-1 as above. Numbers under each figure represent densitometric values that were obtained as described in the legend of Fig. 1, A and B.

Fig. 5. Ectopic expression of c-Rel leads to maintenance of NF-κB transcriptional activity upon TGF-β1 treatment. AML-N1, AML-R2, and AML-R5 cells were treated for 12 h with 2 ng/ml TGF-β1 or BSA-carrier solution and transfected with 20 μg of the E8-CAT reporter construct. Lysates were assayed for CAT activity, as described in the legend of Fig. 1D.

Ectopic Expression of c-Rel Prevents TGF-β1-mediated but not Staurosporine-mediated Apoptosis of AML-12 Cells. The AML-R2 and AML-R5 clones were tested for the extent of apoptosis following TGF-β1 treatment compared to the pSV2Neo-transfected AML-N1 and AML-N2 clones. Loss of cell viability was quantitated by trypan blue exclusion in cultures incubated for 48 h in the presence of TGF-β1 or BSA-carrier solution. TGF-β1 treatment of AML-N1 and AML-N2 clones resulted in a significant loss in viable cells, as noted by the levels of trypan blue-positive cells, 56 ± 8% and 45 ± 6%, respectively (Fig. 6A). In contrast, ectopic c-Rel expression led to a very significant increase in cell survival. Only 18 ± 3% and 15 ± 5% of the AML-R2 and AML-R5 cells, respectively, were trypan blue positive (Fig. 6A). Consistent with these findings, AML-R2 and AML-R5 cells had greatly reduced punctuate staining with propidium iodide or change in cell morphology upon TGF-β1 treatment (Fig. 7 and data not shown).

Next, the AML-N1 and AML-R2 clones were tested for the extent of cell proliferation following TGF-β1 treatment. Cultures incubated for 6, 12, 24, or 48 h in the presence of TGF-β1 or BSA-carrier solution were quantitated by MTS assay. TGF-β1 treatment of AML-N1 cells resulted in a significant block of cell proliferation by 24 h that was more pronounced by 48 h (Fig. 6B), in good agreement with the results obtained above with the parental AML-12 cells (Fig. 2A). In contrast, TGF-β1 treatment had a much more modest effect on cell proliferation of AML-R2 cells. Thus, ectopic c-Rel expression prevents TGF-β1-induced growth arrest and apoptosis of murine hepatocytes.

To determine whether ectopic expression of c-Rel was sufficient to protect AML-12 cells from other inducers of apoptosis, the AML-N1 and AML-R2 clones were tested for cell death following treatment with staurosporine, a broad spectrum inhibitor of protein kinases that has been shown to cause cell death of murine hepatocytes (34). Incubation of AML-N1 cells with 100 nM or 1 μM staurosporine (in DMSO) for 24 h led to a dramatic increase in cell death (Fig. 6C) and growth arrest (data not shown) compared to treatment with DMSO alone, which was more pronounced by 48 h. Furthermore, staurosporine treatment killed the c-Rel-expressing AML-R2 cells to an extent similar to that of AML-N1 cells (Fig. 6C). Thus, staurosporine affects the viability of murine hepatocytes through a pathway independent of NF-κB/Rel factor expression.
NF-κB Inhibition by TGF-β1 Induces Hepatocyte Apoptosis

IκB-α Synthesized at Later Times after TGF-β1 Treatment Is More Stable due to Hypophosphorylation. NF-κB activity is tightly regulated via interaction of its p65 subunit with the inhibitory protein IκB-α in the cytoplasmic compartment (4). Thus, we analyzed the effects of TGF-β1 on expression of IκB-α in the hepatocyte cell lines, using immunoblotting analysis of cytosolic extracts from TGF-β1-treated AML-12 and NMH cells (Fig. 8, A and B, respectively). A slight down-modulation of the IκB-α protein level was noted at the 1-h time point, and then the levels began to increase by 2 h; these higher levels persisted throughout the time course. At the 10-h time point, TGF-β1 treatment increased the levels of IκB-α protein in AML-12 and NMH cells by 2.1- and 1.5-fold, respectively, as judged by densitometric scanning (Fig. 8C). In contrast, no consistent changes in IκB-β protein levels were detected (Fig. 8, A and B and data not shown). Thus, TGF-β1 causes a selective increase in IκB-α protein at later times, when the level of NF-κB binding is reduced. The observation that TGF-β1 treatment for 10 h increased IκB-α protein levels above control values prompted us to analyze whether there was a decrease in turnover of IκB-α protein at these later times. NMH cells were incubated for 12 h in the presence of TGF-β1 or BSA-carrier solution, and then treated with the protein synthesis inhibitor cycloheximide for 2–4 h (Fig. 9A). Cytoplasmic extracts were then subjected to immunoblot analysis for IκB-α. In the BSA-treated cells, IκB-α protein displayed a half-life of approximately 2.3 h as judged by densitometric analysis (Fig. 9B). In the TGF-β1-treated cells, the half-life increased to greater than 4 h (Fig. 9, A and B). In contrast, IκB-β protein has a longer half-life such that no decay was detected over the same 4-h time course with either BSA-carrier solution or TGF-β1 treatment (Fig. 9A). Similar results were found in AML-12 cells (data not shown). Thus, prolonged TGF-β1 treatment of NMH cells stabilizes the IκB-α protein, preventing its normal decay.

Because basal degradation of IκB-α protein requires phosphorylation (4), the phosphorylation levels of the IκB-α protein were analyzed using two-dimensional gel electrophoresis of cytosolic extracts from NMH cells following 10 h of treatment with TGF-β1 or BSA-carrier solution. Cytoplasmic extracts were resolved in isoelectric focusing gels and then separated according to molecular weight by SDS-PAGE. IκB-α protein was detected using immunoblot analysis. Extracts from exponentially growing NMH cells displayed two distinct phosphoisoforms of the IκB-α gene product, migrating in the first dimensional gel between pH 5 and 6 (Fig. 9C, arrowheads). A slower migrating, nonspecific protein was also recognized by the antibody preparation. Hypophosphorylated forms of IκB-α product migrate toward a more basic pH, whereas more negatively charged hyperphosphorylated forms migrate toward a more acidic pH. The presence of phosphorylated forms in BSA-treated cells (43% relative to total protein, as measured by densitometry) is consistent with a constitutive turnover of this inhibitory protein. In the TGF-β1-treated samples, IκB-α was found to be mainly in a hypophosphorylated state when compared to the BSA control (71% versus 57% of total protein, respectively; Fig. 9C). Thus, hypophosphorylation of IκB-α protein in response to TGF-β1 treatment correlates with increased stability of this inhibitor.

Next, we asked whether IκB-α protein stabilization led to enhanced interaction with NF-κB. Extracts from TGF-β1 or

![Figure 6](image-url)

Fig. 6. Ectopic c-Rel increases cell viability and proliferation of AML-12 cells treated with TGF-β1. In A, cultures of clones AML-N1, AML-N2, AML-R2, and AML-R5 were incubated for 48 h in triplicate in the presence of 2 ng/ml TGF-β1 or BSA-carrier solution. Trypan blue-positive cell number was determined and plotted as a percentage of the total cell number. In B, cultures of clones AML-N1 and AML-R2 were incubated for 6, 12, 24, or 48 h in the presence of 2 ng/ml TGF-β1 or BSA-carrier solution. Cell proliferation was quantitated by MTS assay from triplicate samples as described in the legend to Fig. 2A. In C, ectopic c-Rel is not sufficient to rescue AML-12 cells from staurosporine-induced cell death. Cultures of clones AML-N1 and AML-R2 were incubated in duplicate for 24 or 48 h in the presence of 100 nM or 1 μM staurosporine (St.) or DMSO-carrier solution (DMSO) as control. Trypan blue-positive cell number was determined and plotted as a percentage of the total cell number.
BSA-treated NMH cells were immunoprecipitated with an anti-\(\text{IκB-α}\) antibody and then analyzed by immunoblotting for RelA protein. Higher levels of RelA protein were found associated with \(\text{IκB-α}\) in the TGF-β1-treated sample relative to the control (Fig. 9D). Taken together, these findings indicate that TGF-β1 treatment of murine hepatocyte cells decreases nuclear NF-κB levels via production of a more stable, hypophosphorylated form of IκB-α protein that associates with the p65 subunit of NF-κB in the cytoplasm.

**Discussion**

In this study, we have shown that TGF-β1 treatment of AML-12 and NMH untransformed murine hepatocyte cell lines down-modulated the constitutive NF-κB activity while activating a program leading to cellular death. Ectopic expression of c-Rel, which is only expressed at low levels by these cells, rescued AML-12 cells from TGF-β1-mediated cell death. The decrease in NF-κB activity that was apparent by 10 h of TGF-β1 treatment could be correlated with stabilization of \(\text{IκB-α}\) protein due to hypophosphorylation. As a result, RelA is sequestered in the cytosolic compartment, bound to its inhibitory protein, and NF-κB activity was down-regulated. Taken together, these observations demonstrate a pivotal role of NF-κB in mediating signals leading to cell survival of murine hepatocytes, and these observations identify a novel mechanism of action for TGF-β1 that involves tight control of NF-κB and \(\text{IκB-α}\) expression.

In the liver, apoptosis is a physiologically relevant process involved in the normal and preneoplastic turnover of cells (35). TGF-β1 has been implicated previously in the induction of growth arrest and apoptosis of hepatocytes. TGF-β1 treatment induced cell death of primary cultures of rat hepatocytes and cooperated, *in vivo*, with the hepatotoxin cyproterone acetate in the induction of apoptosis of rat liver cells (26). Apoptotic hepatocytes in normal and preneoplastic liver showed immunostaining for TGF-β1 (36). In addition, increased TGF-β1 expression was observed in rat hepatocytes undergoing apoptosis during allogeneic graft rejection (37). Here we found that TGF-β1 treatment of two murine hepatocyte cell lines causes extensive cellular death by apoptosis within 24 h. Similar to what has been reported previously (26), we were unable to detect short, oligosomal DNA fragments in these cells upon TGF-β1 stimulation (data not shown). Therefore, three different criteria were used: MTS conversion to formazan, propidium iodide nuclear staining, and trypan blue exclusion. All of these parameters confirm that AML-12 and NMH cells die from apoptosis in response to TGF-β1.

Fig. 7. TGF-β1-induced apoptosis of AML-12 cells is ablated by ectopic c-Rel expression. Cultures of clones AML-N1 and AML-R2 were incubated in medium containing 2 ng/ml TGF-β1 or BSA-carrier solution for 48 h. AML-N1 and AML-R2 cells were fixed and stained with propidium iodide and photographed at a magnification of ×200 or ×300 (inset) with Tmax film. Arrows, clumps of cells with nuclei displaying extensive chromatin condensation, a hallmark of apoptosis.
Recently, inhibition of NF-κB expression has been linked to apoptosis. Beg et al. (30) showed that RelA-deficient murine embryos die by 14 days of gestation from massive liver degeneration due to hepatocyte apoptosis, although it was unclear whether this effect was direct or indirect. Work from our laboratory has shown that inhibition of NF-κB activity is necessary and sufficient to cause cell death of murine B lymphomas (10, 29). These observations were then extended by other groups to different cell types that do not express constitutive NF-κB/Rel factors (11-14). Here, we have shown that suppression of NF-κB activity is required for TGF-β1-mediated liver cell death. This finding extends to non-lymphoid cells the observation that inhibition of NF-κB/Rel by TGF-β1 induces apoptosis (29). In addition, the finding that ectopic expression of c-Rel is not sufficient to prevent staurosporine-mediated cell death of AML-12 cells suggests the existence of other pathways, independent of NF-κB/Rel factors, leading to the death of murine hepatocytes.

TGF-β1 has been shown to inhibit the induction of DNA synthesis within rat liver cells in vivo following PH (28). Interestingly, a rapid and strong induction of NF-κB binding activity was noted following PH (16, 17). Very recently, mice lacking type I tumor necrosis factor receptor displayed a severely impaired liver regeneration and complete inhibition of NF-κB binding after PH (38). Thus, it is possible that TGF-β1 mediates inhibition of hepatocyte cell proliferation after PH through down-regulation of NF-κB activity. Surprisingly, a major portion of the IκB-α protein at later times after TGF-β1 treatment, presumably newly synthesized, was found to be hypophosphorylated, and this could be correlated with stabilization from its normal turnover. A similar regulation of IκB-β protein phosphorylation levels has been described during LPS stimulation of 70Z/3 pre-B cells (39).

However, in this latter case, hypophosphorylation of newly synthesized IκB-β protein could be associated with persistent NF-κB activation upon LPS exposure. As a result of IκB-α hypophosphorylation upon TGF-β1 treatment, higher levels of RelA protein were found to be associated with IκB-α in the cytosolic compartment, therefore leading to persistent inactivation of NF-κB activity. At early times, TGF-β1 treatment resulted in a transient down-modulation of IκB-α protein and increase in nuclear NF-κB levels. Thus, the mechanism of TGF-β1-mediated events may involve regulation of IκB-α in two phases: early degradation and later stabilization. The constitutive phosphorylation of the COOH-terminal PEST region by casein kinase II has been implicated in the regulation of basal phosphorylation of IκB-α protein (40). Thus, it is tempting to speculate that prolonged TGF-β1 treatment might lead to inhibition of IκB-α-κinase activity such as CK II or to the activation of specific phosphatases. This in turn might affect basal levels of phosphorylation of the IκB-α protein that leads to its stabilization at later times. The activation of NF-κB at early times raises the intriguing possibility that TGF-β1-induced phosphorylation of IκB-α protein is a prerequisite for its degradation and transient NF-κB translocation. Work is in progress to elucidate the signaling pathway that leads to the activation of the IκB-α kinase by TGF-β1.

Materials and Methods

Cell Culture and Treatment Conditions. NMH and AML-12 cells (19) were maintained in DMEM (Ham’s) F-12 medium (Life Technologies, Inc., Gaithersburg, MD), supplemented with 5 ng/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium, 120 μg/ml nicotinamide (all from Sigma Chemical Co., St. Louis, MO), 50 units/ml penicillin, and 50 μg/ml streptomycin. NMH cells were supplemented with 20 ng/ml epidermal growth factor (Collaborative Research, Bedford, MA). AML-12 cells were maintained with 10% fetal bovine serum (Life Technologies, Inc.). For treatment, cells were incubated for the indicated periods of time with 2 ng/ml TGF-β1 (Austral Biological, San Ramon, CA) dissolved in 0.1% carrier BSA or 0.1% BSA as control. Alternatively, cells were incubated with 100 nM or 1 μM staurosporine (Calbiochem-Novabiochem International, San Diego, CA) dissolved in 100% DMSO or the equivalent volume of 100% DMSO as control. To prevent protein synthesis, cells were treated with 10 μg/ml cycloheximide (Sigma Chemical Co.).

Transfection Conditions. For transient transfection, AML-12 cells were resuspended in DMEM/Ham’s F-12 supplemented with 20% FCS
Fig. 9. Prolonged TGF-β1 treatment of NMH cells leads to increased stability and hypophosphorylation of IxB-α protein. In A and B, TGF-β1 treatment reduces the normal turnover of the IxB-α protein. NMH cells were treated for 12 h with 2 ng/ml TGF-β1 (T) or BSA-carrier solution (B) and then incubated in the presence of 20 µg/ml cycloheximide (CHX) for the indicated time points (hr). Cytoplasmic extracts (40 µg) were then subjected to immunoblot analysis using antibody preparation against IxB-α and IxB-β proteins (A). The IxB-α blot was then subjected to quantitation by densitometric scanning, and the results were plotted (B) as a percentage of the initial value (set at 100%) over time. In C, prolonged exposure to TGF-β1 leads to hypophosphorylation of the IxB-α gene product. Cytoplasmic extracts were isolated from NMH cells that were treated with 2 ng/ml TGF-β1 or BSA-carrier solution for 12 h. Samples (40 µg) were then subjected to two-dimensional gel electrophoresis followed by immunoblot analysis, using an antibody preparation against IxB-α. The horizontal axis represents the first dimensional separation. Left, basic values (−); right, acidic values (+). The vertical axis represents the second dimensional separation. Arrowheads, the two most abundant phosphoisoforms of IxB-α. In D, higher levels of p65 are associated with IxB-α in response to TGF-β1 treatment. Cytoplasmic extracts (80 µg) obtained from NMH cells that have been treated for 12 h with 2 ng/ml TGF-β1 or BSA-carrier solution were immunoprecipitated with protein A-Sepharose in the absence (−) or in the presence (+) of an antibody against IxB-α (αIxB-α). Immunoprecipitated proteins were then analyzed by immunoblotting for p65 expression.

EMSA. The URE oligonucleotide (5’-AACTCCGGTTTTCCCAACCG-3’) was end-labeled with Klenow DNA polymerase and α-32P-labeled deoxynucleotide triphosphates and used in EMSA, as described previously (29). Nuclear extracts were prepared from AML-12 and NMH cells by the method of Strauss and Varshavsky, as described elsewhere (31). The binding reaction for octamer-1 was performed with a double-stranded oligonucleotide with the following sequence: 5’-TGTGAATTGCACATAGAG-3’. The underlined regions indicate the core NF-κB and octamer-1 elements. Quantitation by densitometry was performed using a Molecular Dynamic 300A computing densitometer.

Immunoblot Analysis. For isolation of cytoplasmic proteins, washed cells were resuspended in cold 10 mM Tris (pH 7.4), 10 mM NaCl, 3 mM MgCl2, 1 mM EDTA, 0.2 mg/ml phenylmethylsulfonylfluoride, 0.1% SDS, and protein was extracted as described previously (29). For two-dimensional gel electrophoresis, samples (20–40 µg) were separated according to isolectric point by isoelectric focusing in the first dimension, using a mixture of Ampholine (2% w/v; Bio-Rad Laboratories, Hercules, CA) with a pH range of 5–7 and 3–10, as described by O’Farrell (42). Cytoplasmic extracts (80 µg) were immunoprecipitated following the protein A-Sepha-

at a concentration of 16 × 10⁶ cells/ml. Alternatively, NMH cells were resuspended in DMEM (Ham’s) F-12 supplemented with 1.5% BSA at a concentration of 16 × 10⁶ cells/ml. Cells (250 µl) were preincubated on ice for 10 min with DNA (up to 40 µg) and transfected by electroporation at 240 V and 960 µF, as described previously (41). After incubation on ice for 5 min, the cell suspension was mixed with 10 ml of complete medium and incubated for 10 min at room temperature. The suspensions were then transferred to Petri dishes and incubated for 8 h at 37°C. Cells were harvested, and the resulting extracts were normalized for total protein content and β-galactosidase expression, as described previously (41). Equal amounts of lysates were incubated in duplicate with 2.5 µCi [3H]acetyl CoA (DuPont New England Nuclear, Boston, MA), 50 µg acetyl CoA, and 1.6 mV chloramphenicol for 4–6 h, and the acetylated forms were extracted with ethyl acetate and assayed by liquid scintillation. Alternatively, normalized extracts were assayed for luciferase activity, following kit instructions (Promega Corp., Madison, WI). SD was obtained using Student’s t test. The URE-TK-CAT plasmid (EB-CAT) and its double mutated version (dmEB-CAT) have been described previously (31). The pGL2 promoter DNA vector (pGL2-SV40) was obtained from Promega.

The AML-12 c-Rel stable transfecants were prepared using 38 µg of murine c-Rel expression vector (33) and 2 µg of PSV2Neo DNA. After 24 h, 0.66 mg/ml G418 (Life Technologies, Inc.) was added to the medium, and selective growth conditions were maintained for 2 weeks. Clones were isolated using cloning rings.
rose protocol, described previously (43). Isoelectric focusing gels, immuno-precipitants, or crude cytoplasmic extracts (20–40 μg) were then subjected to electrophoresis on a 10% polyacrylamide-SDS gel and transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA); immunoblotting was performed, as described previously (29). The antibody preparation for IxB-β (SC-945), IxB-α (SC-371), and c-Rel (SC-070) were purchased from Santa Cruz Biotechnology, Inc. The antibody preparation for p65 (1226) was provided by Nancy Rice (National Cancer Institute, Frederick, MD).

Apoptosis Assays. For propidium iodide staining, cells were washed three times in PBS and then fixed for 15 min at room temperature in 3.7% formaldehyde. Following incubation with 10 μg/ml propidium iodide (Sigma), 0.1% Triton X-100, and 50 μg/ml RNase A in PBS for 15 min, cells were visualized on a Nikon Optiphot fluorescence microscope, and fluorescent images were recorded at ×200 using Kodak Tmax 3200 film. For trypan blue exclusion assays, cells were incubated with 0.2% trypan blue (Life Technologies, Inc.) for 10 min, and the percentage of cells excluding dye (viable cells) or staining positive (dead cells) was determined. For the Non-Radioactive Cell Proliferation assay (Promega), cells were seeded at 20 × 10^3 in 100-μl volume in 96-well dishes. Cells were incubated in triplicate for 4 h at 37°C in the presence of MTS solution (333 μg/ml) and 25 μM phenazine methosulfate according to the manufacturer’s directions. The A590 was measured in an ELISA plate reader.

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References