Tumor Necrosis Factor Induces DNA Replication in Hepatic Cells through Nuclear Factor κB Activation

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Abstract

Tumor necrosis factor (TNF) signaling through TNF receptor 1 (TNFR1) with downstream participation of nuclear factor κB (NFκB), interleukin 6 (IL-6), and signal transducers and activators of transcription 3 (STAT3) is required for initiation of liver regeneration. It is not known whether the proliferative effect of TNF on hepatocytes is direct or requires the participation of Kupffer cells, the liver resident macrophages. Moreover, it has not been determined whether NFκB activation is an essential step in TNF-induced proliferation. To answer these questions, we conducted studies in LE6 cells, a rat liver epithelial cell line with hepatocyte progenitor capacity. We report that TNF induces DNA replication in growth-arrested LE6 cells and that its effect involves the activation of NFκB and STAT3 and an increase in c-myc and IL-6 mRNAs. All of these effects, which mimic the events that initiate liver regeneration in vivo, are blocked if NFκB activation is inhibited by expression of a dominant-inhibitor IkBα mutant (ΔN-IκBα). Although NFκB blockage by ΔN-IκBα causes caspase activation and massive death of cells stimulated by TNF, inhibition of NFκB and STAT3 binding by the serine protease inhibitor N-tosyl-L-phenylalanine chloromethyl ketone results in G0-G1 cell cycle arrest without death. We conclude that NFκB is an essential component of the TNF proliferative pathway and that TNF-induced changes in IL-6 mRNA, STAT3, and c-myc mRNA are dependent on NFκB activation. Blockage of NFκB inhibits TNF-induced proliferation but does not necessarily cause cell death.

Introduction

TNF3 is a pleiotropic cytokine that participates in a wide range of biological activities, including inflammation, growth, differentiation, and apoptosis. TNF is a key molecule in the cytokine network, capable of regulating the expression of other cytokines, such as IL-6. It is produced as a membrane-bound protein and is released in the serum after being processed into the mature soluble form (1). The cytokine exerts its effects by binding to two distinct receptors, TNFR1 and TNFR2, which are ubiquitously expressed (2). Signaling through TNFR1 is responsible for most of the TNF effects, including activation of the transcription factor NFκB and apoptosis (3). TNFR2 appears to be the main receptor for membrane-bound TNF and for TNF effects on thymocyte proliferation.

TNF is a mediator of the hepatic acute phase response to inflammation (1). However, experiments in which hepatocyte DNA synthesis after PH was inhibited in livers of rats pre-treated with anti-TNF antibodies indicated that TNF may also have a role in hepatocyte proliferation (4). Using mice deficient in TNFR1 or TNFR2, we found that TNF signaling through TNFR1, but not TNFR2, is required for the initiation of liver regeneration after PH (5, 6). The drastically reduced DNA synthesis in the liver remnant of TNFR1 knockout mice was associated with low levels of IL-6, along with decreased activation of the transcription factors NFκB, STAT3, and AP1. Activation of these factors after PH primes quiescent hepatocytes to make them competent to proliferate. In TNFR1 knockout mice, normal DNA synthesis and STAT3 activation but not NFκB binding were restored by a single injection of IL-6 shortly before PH. Cressman et al. (7) showed that IL-6 knockout mice have decreased activation of STAT3 and AP1, diminished cyclin D1 expression, impaired DNA synthesis, and high mortality after PH. Injection of IL-6 corrected all defects and restored the normal proliferative response in these animals (7). NFκB activation after PH was normal in IL-6 knockout mice and did not change after IL-6 injection.

Together, the results from studies of IL-6 and TNFR1 knockout mice suggest that hepatocyte proliferation during liver regeneration can be triggered by a signal transduction pathway that involves TNFR1, NFκB, IL-6, and STAT3. Although these and other reports demonstrated that TNF causes NFκB and STAT3 activation in the liver, it is not known whether NFκB activation is required for DNA replication and to what extent IL-6 production and STAT3 binding in the liver relate to NFκB activity. Moreover, it has been suggested that the TNF effect on hepatocyte proliferation might be indirect, requiring the involvement of Kupffer cells. TNF could induce hepatocyte replication by a two-step process,

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3 The abbreviations used are: TNF, tumor necrosis factor; TNFR, TNF receptor; IL, interleukin; NF, nuclear factor; PH, partial hepatectomy; STAT, signal transducers and activators of transcription; AP, activating protein; TPCK, N-tosyl-L-phenylalanine chloromethyl ketone; BrdUrd, bromodeoxyuridine; EMSA, electrophoretic mobility shift assay; CHX, cycloheximide; wt, wild type; OSM, oncostatin M.
the first being its acting on Kupffer cells to activate NFκB and produce IL-6. The second step would consist of the binding to hepatocyte receptors of IL-6 produced by Kupffer cells, causing STAT3 activation and subsequent hepatocyte proliferation.

To determine whether TNF can directly, without the mediation of Kupffer cells, stimulate liver cell replication through a NFκB-mediated pathway, we conducted studies in LE6 rat liver epithelial cells. These cells, which have hepatocyte progenitor capacity, are not transformed and can be growth arrested in low serum and used for growth re-initiation studies (8). We report that a short exposure to TNF causes a proliferative response in growth-arrested LE6 cells. This effect involves the activation of NFκB and the sequential increase in IL-6 mRNA and STAT3 activation, mimicking the events that occur at the initiation of liver regeneration in vivo. In addition, TNF caused an increase in c-myc mRNA, again in similarity with the regenerating liver. Blockage of NFκB activity by a dominant ΔN-IkBα gene reduced TNF-mediated increases in c-myc and IL-6 mRNAs, inhibited STAT3 activation, and abrogated TNF-induced DNA synthesis, leading to massive apoptotic cell death. Inhibition of NFκB by TPCK, a nonspecific NFκB inhibitor, also led to blockage of TNF-induced STAT3 binding and IL-6 mRNA accumulation, causing cell cycle arrest without apoptosis.

Results

TNF Can Reinitiate DNA Replication in Growth-arrested LE6 Cells. Given the importance of TNF for the initiation of liver regeneration, we first determined whether TNF could directly stimulate DNA synthesis in LE6 cells. Cells were growth arrested by culturing in 0.5% FCS for 3 days (Fig. 1A). Short-term (30-min) exposure of growth-arrested LE6 cells to recombinant human TNF in the absence of FCS, followed by incubation in 0.5% FCS, increased the proportion of BrdUrd-positive cells to 20–30%, with a peak at 16–20 h after treatment (Fig. 1A). Data shown in Fig. 1B examine some of the features of the TNF proliferative effect. Cells were exposed to TNF in serum-free medium for 30 min, after which the medium was changed to contain 0.5% FCS, 10% FCS, or no serum. For comparative purposes, the medium in some culture dishes was changed from 0.5 to 10% FCS without prior TNF exposure. BrdUrd was added to all cultures 14 h after medium change, and the experiments terminated 4 h later. In the absence of TNF, DNA labeling in growth-arrested cells increased by ~3-fold by addition of 10% serum (Fig. 1B, second column). Cells exposed to TNF showed no increased in DNA replication in serum-free medium, but proliferation was greatly increased in cells maintained in 0.5 or 10% FCS (approximately 6- and 8-fold increase, respectively, compared with growth-arrested cells). Continuous treatment by TNF for 18 h in cells maintained in 0.5% serum did not result in higher DNA replication than a 30-min TNF exposure (not shown). We conclude that a short pretreatment of growth-arrested LE6 cells primes the cells to undergo DNA replication, and that the priming effect requires the presence of serum.

Activation of NFκB and STAT3 by TNF. During liver regeneration, TNF signaling involves NFκB activation, IL-6 production, and STAT3 activation. Our aim was to find out whether these factors may be activated in hepatic LE6 cells in response to TNF. DNA binding activities of the transcription factors NFκB and STAT3 were evaluated by EMSA. Growth-arrested cells had a low basal level of NFκB activity (Fig. 2A, Lanes 1 and 2), which was greatly increased upon TNF stimulation. Binding reached a peak 15 min (Fig. 2A, Lane 3) after the end of TNF exposure and declined within the next 2 h (Fig. 2A, Lanes 4 and 5). Mobility-shift assay, performed after preincubation of nuclear extracts with antibodies specific to p65 and p50 proteins, revealed that TNF-stimulated binding complexes were composed of p50/p65 heterodimers (NFκB) and p50 homodimers (Fig. 2B), as is the case for regenerating liver.

Changes in STAT3 binding after TNF treatment occurred ~1 h later than NFκB activation. TNF caused an increase in STAT3 binding at 30–60 min after exposure (Fig. 2C), but it was no longer detectable at 2 h. To exclude the possibility that the STAT3 increase was caused by serum rather than TNF, cells were maintained in serum-free medium after TNF exposure. Increased binding of STAT3 in cells pretreated with TNF was similar in cells kept in serum-free or serum-containing medium (Fig. 2C). Activation of STAT3 by TNF required protein synthesis (Fig. 2D) because it could be blocked by CHX. In contrast, STAT3 activation by IL-6 appeared to be direct because it occurred rapidly (10–15 min after treatment) and was not inhibited by CHX (Fig. 2D). In summary, exposure of growth-arrested LE6 cells to TNF led to activation of NFκB within 15 min, followed by activation of STAT3, which reached its peak 1 h after TNF treatment.

Specific Inhibition of NFκB Activity by Inducible Expression of ΔN-IκBα Gene Decreases TNF-induced STAT3 Activation. To determine the role of NFκB in TNF-induced proliferative pathways in LE6 cells, NFκB activity was specifically blocked by overexpression of a phosphorylation, and degradation impaired NFκB inhibitor IκBα mutant (ΔN-IκBα). The expression plasmid is driven by a zinc-inducible promoter (MRE) to switch on transgene expression. This plasmid was transfected into LE6 cells to establish stable clonal cells referred to as LE6-9 cells. These cells were maintained in medium without zinc and exposed to zinc sulfate to activate expression of the constructs [these conditions will be referred to as LE6-9(+Zn) or (+Zn) cells]. Expression of wt IκBα and ΔN-IκBα was analyzed by Western blot in LE6-9 cells as well as in pooled stable clones of cells transfected with a MRE-pNeo vector that did not contain the ΔN-IκBα gene (Fig. 3A). A single M, 37,000 band corresponding to wt IκBα was detected in extracts of LE6-C cells with or without zinc and in LE6-9(−Zn) cells. Activation of the MRE promoter with zinc in LE6-9 cells [LE6-9(+Zn)] caused the appearance of a lower molecular weight band corresponding to ΔN-IκBα (Fig. 3), whereas the wt IκBα band was no longer detectable (Fig. 3A). Loss of wt IκBα in LE6-9(−Zn) cells might be attributable to its short life. Furthermore, because IκBα is one of the NFκB target genes (9–11), overexpression of ΔN-IκBα may prevent NFκB-mediated re-synthesis of IκBα.

In LE6-C cells, TNF exposure increased NFκB binding in the presence or absence of zinc (Fig. 3B, Lanes 3 and 4),
whereas zinc by itself had no detectable effect on the binding. TNF also stimulated NFκB binding in LE6-9(−Zn) cells (Fig. 3B, Lanes 5 and 7), but the stimulation was greatly diminished in LE6-9(−Zn) cells, which express the ΔN-IXBα protein (Fig. 3B, Lane 8). Similarly, TNF activated STAT3 in LE6-C(−Zn) and (−Zn) cells (Fig. 3C, Lanes 1–4) as well as in LE6-9(−Zn) cells (Fig. 3C, Lane 7). However, STAT3 induction by TNF was greatly diminished in LE6-9(−Zn) cells (Fig. 3C, Lane 8). In summary, the results demonstrate that TNF-induced NFκB binding can be blocked by ΔN-IXBα and that NFκB blockage inhibits STAT3 activation.

**The Pathway Leading from TNF to DNA Synthesis Is Changed to a Death Pathway upon Inactivation of NFκB by ΔN-IXBα.** LE6-9(−Zn) cells were growth arrested by maintaining them in 0.5% FCS for 3 days. Under those conditions, ~5% of cells were stained by BrdUrd. Exposure of growth-arrested LE6-9(−Zn) cells to TNF increased DNA replication by 5–7-fold. The magnitude of the increase was similar for TNF-stimulated parental LE6 cells in presence or absence of zinc. However, exposure of LE6-9(−Zn) cells to TNF resulted not in proliferation but instead caused massive cell death. Ten h after TNF treatment, ~60% of cells were killed, as demonstrated by flow cytometric analysis, and virtually all cells died by 14 h (Fig. 4). Thus, TNF acts as a strong killing agent rather than a mitogen in LE6 cells in which NFκB binding (and as a consequence, STAT3 activation) is blocked. Similar results have been obtained in differentiated mouse hepatocytes,4 in a hepatocyte cell line as well as other cell types (12–14).

**Inhibition of NFκB and STAT3 Activity by TPCK Pretreatment Blocks TNF-induced DNA Synthesis in LE6 Cells But Does Not Lead to Cell Death.** Because blockage of NFκB by the inducible ΔN-IXBα gene caused massive cell death, we looked for an alternative method to study the relationships between NFκB activation and DNA synthesis in TNF-stimulated cells. A widely used NFκB blocking agent, the severe protease inhibitor TPCK (15, 16), inhibits phosphorylation and degradation of IXBα. It has also been reported that TPCK may have antiapoptotic effects (17). We determined whether TPCK would block NFκB in LE6 cells and whether this blockage would have a similar effect as NFκB blockage by ΔN-IXBα. TPCK exposure for 1.5 h had no effect on the viability of LE6 cells maintained in 0.5% FCS for 3 days. Addition of TNF to growth-arrested LE6 cells pretreated by TPCK for 1 h abolished TNF-induced DNA synthesis. However, the cells remained viable and morphologically intact for at least 5 days (Fig. 5A). Flow cytometric analysis confirmed and extended the morphological observations and the data obtained by BrdUrd staining. TNF treatment of growth-arrested LE6 cells increased by ~2-fold the percentage of cells in S phase, whereas the number of cells in G2–M phase declined (Fig. 5, B and C; Table 1). TPCK pretreatment before TNF exposure blocked the elevation in S-phase cells and increased the proportion of cells in G2–M (Fig. 5D; Table 1). An examination of the flow cytometric profiles shown in Fig. 5 confirmed previous observations that

4 M. Chaissen and N. Fausto, unpublished observations.
LE6 cells are predominantly diploid. However, because the population contains a small amount of tetraploid cells, it is difficult to estimate the percentage of diploid cells in G2 (the G2 peak of diploid cells may overlap with G0-G1 peaks of tetraploid cells). Consistent with the lack of morphological signs of cell death in LE6 cells exposed to TNF and pre-treated with TPCK, no pre-G0-G1 peak characteristic of apoptosis was detectable in the cells by flow cytometry (Fig. 5, B–D).

The effect of TPCK on TNF-induced NFκB and STAT3 binding was analyzed by EMSA (Fig. 6). NFκB binding was strongly inhibited (Fig. 6A, Lanes 4 and 5), and STAT3 binding was not detectable (Fig. 6B, Lane 6) in TNF-exposed cells pretreated with TPCK. To determine whether the lack of STAT3 activation resulted from the blockage of NFκB activity or from direct inhibition of STAT3 binding, cells were treated for 15 min with 2 ng/ml of IL-6. The direct activation of STAT3 by IL-6 was abolished by a 1-h pretreatment of the cells with TPCK (Fig. 6B, Lanes 4, 5, and 7). Moreover, the addition of IL-6 to TNF-treated cells prouncubated with TPCK failed to restore STAT3 binding (not shown). Thus, TPCK may have a direct effect on STAT3 activity, probably by blocking its phosphorylation as it does for IκBα (15, 16).

In summary, the results show that TPCK blocks TNF-mediated binding of both NFκB and STAT3 and inhibits DNA replication by G0-G1 growth arrest without killing the cells. In contrast, NFκB and STAT3 blockage by ΔN-IκBα causes massive cell death after TNF exposure. A major feature of TNF-mediated apoptotic cell death is the activation of caspase (18). We determined whether caspase-3 activity would differ in cells treated with TNF in which NFκB and STAT3 binding was blocked by either the expression of ΔN-IκBα or pretreatment by TPCK. Parental LE6 cells had a low level of caspase-3 activity, which was not increased after TNF exposure with or without TPCK pretreatment (Fig. 7). In contrast, blockage of NFκB by ΔN-IκBα in TNF-treated LE6-9(-Zn) cells caused a 2.5-fold increase in caspase-3 activity. TPCK did not block caspase-3 activity in LE6-9(-Zn) cells and did not protect these cells from apoptotic cell death, indicating that it does not have a direct inhibitory effect on caspase-3 activity.

**LE6 Cells Accumulate IL-6 mRNA in Response to TNF.** In the regenerating liver of TNFR1-deficient mice, binding of both NFκB and STAT3 fail to increase after PH and IL-6 levels are low. In these animals as well as in IL-6 knockouts, IL-6 injection before PH restores STAT3 but not NFκB binding in the regenerating liver, indicating that it does not have a direct inhibitory effect on caspase-3 activity.

![Fig. 2. EMSA of NFκB (A and B) and STAT3 (C and D) in growth-arrested LE6 cells treated with TNF. A, growth-arrested cells were treated with TNF for 30 min in the absence of FCS, after which the medium was changed to 0.5% FCS. Nuclear protein extracts were prepared from growth-arrested cells (Lanes 1 and 2) and after 15–120 min after TNF treatment (Lanes 3–6). NFκB, p50/p65 heterodimer; (p50)2, p50/p50 homodimer. B, supershift analysis of NFκB DNA binding was carried out using specific p65 and p50 antibodies (ab; Santa Cruz). Five μg of nuclear protein prepared from TNF-treated LE6 cells were incubated for 30 min with the NFκB probe, followed by the addition of 1 μg of antibody for the next 30 min. C, STAT3 binding of growth-arrested LE6 cells (Lanes 1 and 2) and after 15 and 60 min after TNF treatment (Lanes 3–6, cells in 0.5% serum; Lanes 7–8, cells maintained in serum-free medium). D, effect of CHX treatment (10 and 50 μg/ml) on STAT3 binding induced by TNF (Lanes 2–4) and IL-6 (Lanes 5–7). These results are representative of three independent experiments.](image)
the ΔN-IkBα gene (Fig. 8, Lanes 5–7). TNF induction of IL-6 mRNA in parental LE6 cells was also blocked by TPCK treatment (not shown). These data demonstrate that LE6 cells produce IL-6 mRNA in response to TNF and that the induction is NFκB dependent. However, we do not know whether IL-6 (protein) is produced because we failed to detect IL-6 in LE6 cultures by means of ELISA, Western Blots, or by using neutralizing antibodies. We also investigated whether IL-6, like TNF, would have a proliferative effect on growth-arrested LE6 cells. Recombinant human or rat IL-6 in a wide concentration range (from 10 pg/ml to 100 ng/ml) had no effect on DNA replication in growth-arrested LE6 cells maintained in 0.5% FCS. Recombinant IL-6 also did not enhance DNA replication in cultures maintained in FCS concentrations ranging from 1 to 10%. IL-6 added together with TNF or 2 h before TNF exposure did not prevent cell death or restore the proliferative pathway in LE6-9(+Zn) cells, which have NFκB blockage caused by IkBα expression. In summary, TNF induction of IL-6 mRNA in LE6 cells is NFκB dependent, but we could not demonstrate the presence of IL-6 or show that recombinant IL-6 would stimulate DNA replication.

c-myc Is One of the TNF-induced NFκB Targets. Accumulation of c-myc RNA, a cell cycle gene with multiple functions (19), occurs 1 h after PH (20) after NFκB activation. NFκB was shown to directly participate in induction of c-myc transcription in fibroblasts and T-cell and B-cell lymphomas (21, 22). We determined whether c-myc is a target gene for TNF-activated NFκB in LE6 cells. In growth-arrested LE6-9 and LE6-C cells, c-myc was weakly expressed (Fig. 8B, Lanes 1 and 5), and the expression was not significantly influenced by the addition of zinc sulfate (Fig. 8B, Lanes 3 and 7). TNF treatment strongly elevated c-myc RNA levels in LE6-C (–Zn) or (+Zn) cells as well as in LE6-9(–Zn) cells. However, c-myc mRNA induction by TNF was greatly diminished in LE6-9(+Zn) cells in which NFκB binding is blocked (Fig. 8B, Lane 8). As shown above, TNF-mediated STAT3 activity is NFκB dependent as well. However, the peak of STAT3 binding takes place 1 h after the end of TNF exposure, whereas c-myc RNA accumulates 20 min after the exposure, after NFκB activation but before STAT3 activation. Moreover, IL-6 does not increase c-myc mRNA levels (data not shown), although it induces STAT3 activation. This ob-
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**Discussion**

Signaling through TNFR1, involving NF\textsuperscript{k}B activation, IL-6 production, and STAT3 binding, is necessary for the initiation of liver regeneration after PH (5) or chemical injury (23). These in vivo experiments did not address the question of whether TNF acts directly on hepatocytes to stimulate this pathway or whether its primary targets are mesenchymal cells such as Kupffer cells, which can produce IL-6 as a result of NF\textsuperscript{k}B activation. If the last possibility were correct, both parenchymal and nonparenchymal cells would be needed to reconstitute in culture the TNF signaling pathway. Other important questions not directly addressed by the mouse experiments were whether TNF-induced NF\textsuperscript{k}B is indeed the first step in a sequence of events that leads to hepatocyte proliferation and what impact the specific blockage of NF\textsuperscript{k}B would have on subsequent STAT3 activation and DNA replication.

We have used hepatocytes in primary culture as well as liver cell lines to study some of these questions. The major limitation of primary cultures is that many of the early events that initiate liver regeneration, including the activation of proto-oncogenes and transcription factors, are triggered by the collagenase digestion step needed for hepatocyte isolation. Furthermore, because hepatocytes in primary culture are short-lived, it is not possible to use them for experiments involving stable transfections. For these reasons, we used LE6 cells, a nontumorsformed, nontumorigenic liver epithelial cell line, to determine whether TNF has a direct proliferative effect on hepatic cells through a pathway that involves NF\textsuperscript{k}B.

Here we show that TNF induces DNA replication in growth-arrested LE6 cells and that a short pretreatment of the cells is sufficient to produce this effect. As has been observed in hepatocytes in primary culture,\textsuperscript{5} TNF only induces DNA synthesis in liver cells in the presence of serum and is inactive in serum-free cultures. These findings indicate that TNF is not a complete mitogen for liver cells but that it acts by “priming” liver cells for replication. Similarly to the TNF-activated cascade that occurs shortly after PH, the TNF-induced proliferative pathway in LE6 cells involves NF\textsuperscript{k}B activation, increases in IL-6 mRNA and c\textsuperscript{\text{-}myc} mRNA levels, and STAT3 activation. By blocking NF\textsuperscript{k}B, we demonstrated that IL-6 mRNA and c\textsuperscript{\text{-}myc} mRNA accumulation, STAT3 activation, and DNA replication depend on NF\textsuperscript{k}B activation. As a result of specific NF\textsuperscript{k}B blockage by the inducible expression of \( \Delta \text{N-I}\text{k}B\alpha \), the TNF-induced proliferative pathway was switched to an apoptotic one. Blockage of NF\textsuperscript{k}B and STAT3 by the serine protease inhibitor TPCK abrogated TNF-initiated DNA replication as well but arrested cells in the G\text{0}-G\text{1} phase without apoptosis.

NF\textsuperscript{k}B activation in LE6 cells by TNF is rapid and is followed by STAT3 activation. STAT3 activation is indirect because it was inhibited by CHX and decreased by specific blockage of NF\textsuperscript{k}B activation. Expression of \( \Delta \text{N-I}\text{k}B\alpha \) had no effect on the low basal levels of STAT3 binding detected in growth-arrested cells and did not interfere with IL-6-induced STAT3 activation. We conclude that TNF-induced STAT3 activation in these cells is dependent on NF\textsuperscript{k}B activity. On the basis of the data obtained with TNFR1 and IL-6 knockout mice (5, 7), we hypothesized that IL-6 is an NF\textsuperscript{k}B downstream product (24), and a potent STAT3-inducing agent (25), would also mediate TNF-induced STAT3 activation in LE6 cells. It is generally assumed that IL-6 in the liver is synthesized by Kupffer cells, the hepatic resident macrophages (26). However, it has also been reported that rat hepatocytes can produce IL-6 in response to lipopolysaccharide (27, 28). Our data demonstrate that TNF increases IL-6 mRNA levels in growth-arrested LE6 cells and that the increase is blocked by expression of \( \Delta \text{N-I}\text{k}B\alpha \). However, we could not detect IL-6 protein in the cultures, perhaps because it is produced in very small amounts or is rapidly degraded. We explored the possibility that growth factors and other IL-6 family cytokines might activate STAT3 (25) LE6 cells. Leukemia inhibitory factor had little effect on STAT3 activation and predominantly activated STAT1 homodimers, but OSM strongly activated STAT3 binding. Neither transforming growth factor \( \alpha \), nor epidermal growth factor, potential STAT3-activating agents (25), had major effects on STAT3 activation. In any event,

\[5\] Y. Yamada and N. Fausto. Effects of TNF and IL-6 in primary cultures of hepatocytes from normal and TNF receptor knockout mice, manuscript in preparation.
IL-6 or perhaps OSM can participate in a NFκB-dependent pathway by which TNF activates STAT3. It is of interest that this pathway is active in liver epithelial cells without requirement of the participation of Kupffer cells or other nonparenchymal cells. This finding, however, does not exclude a role for other cell types in enhancing this and other parallel TNF-induced signaling pathways during liver regeneration. A single injection of IL-6 restored DNA replication of regenerating livers in hepatocytes of TNFR1 knockout mice, but it did not induce DNA replication in normal (non hepatocellular) mice (5). In LE6 cells, IL-6 (as well as OSM and leukemia inhibitory factor) failed to stimulate DNA replication.

Table 1  Effect of TPCK on TNF-induced DNA synthesis in LE6 cells

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<tr>
<th>Condition</th>
<th>S phase</th>
<th>G2–G1 phase</th>
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<tr>
<td>0.5% FCS</td>
<td>13.6 ± 0.5</td>
<td>64.5 ± 4.5</td>
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<tr>
<td>TNF</td>
<td>28.3 ± 2.5</td>
<td>55.4 ± 3.2</td>
</tr>
<tr>
<td>TPCK + TNF</td>
<td>15.1 ± 1.6</td>
<td>73.7 ± 2.5</td>
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* Percentage of cells in S or G2–G1 phase measured by flow cytometry. The values are the means ± SD, obtained from data shown in Fig. 5.

It is logical to assume that TNF-induced NFκB activates multiple pathways required for cell cycle progression. We show that TNF increases the level of c-myc mRNA in LE6 cells, and that specific blockage of NFκB activity inhibits this effect. Transactivation of c-myc by NFκB has been demonstrated in fibroblasts as well as in B- and T-cell lymphomas (21, 22). In some cell types, the “inappropriate” overexpression of c-myc can promote apoptosis, particularly upon withdrawal of growth-promoting signals. In LE6 cells, c-myc mRNA accumulation occurred in response to TNF and was associated with TNF-induced proliferation. The increase was abrogated during TNF-triggered apoptosis, suggesting that c-myc is one of the NFκB-induced protective genes, as has been described in B-cell lymphomas (29).

Blockage of NFκB by ΔN-ΙκBα or TPCK revealed that its activation is essential for TNF-induced STAT3 activation, IL-6 mRNA accumulation, and DNA synthesis in LE6 cells. However, ΔN-ΙκBα expression increased caspase-3 activity and caused massive apoptosis in response to TNF, whereas TPCK treatment blocked TNF-induced proliferation, arresting cells in G0–G1 without caspase activation or cell death. TPCK might have antiapoptotic effects by blocking caspase activity, but it did not block caspase-3 activation in cells undergoing apoptosis resulting from ΔN-ΙκBα expression. Preliminary experiments suggest that TPCK may enhance...
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TNF-induced AP-1 and c-Jun NH₂ kinase activity, which may protect cells from apoptosis, as demonstrated in other systems (30).

In summary, the study shows that the TNF-activated pathway involved in the initiation of liver regeneration functions in epithelial hepatic cells without participation of other cell types and that TNF-induced DNA replication as well as STAT3 binding require NF-κB activation.

Materials and Methods

Cell Lines. The rat nontransformed liver epithelial cell line LE6 was derived from oval cells, which proliferate in the livers of animals maintained for 6 weeks on a choline-deficient diet, supplemented with ethionine for 6 weeks (31). These cells are non tumorigenic when injected s.c. or in the liver of nude mice and do not grow in soft agar. LE6 cells grow in medium supplemented with 10% FCS and differentiate in hepatocytes if maintained in three-dimensional cultures. Reducing FCS content to 0.5% for 3 days causes growth arrest of confluent cultures.

Stable Transfections. Murine IκBα cDNA was obtained from Paul Noble (Johns Hopkins, Baltimore, MD). A phosphorylation- and degradation-impaired IκBα deletion mutant (ΔN-IκBα), lacking amino acids 1–36, was constructed and subcloned into the MRE-pNeo vector as described previously (32). The plasmid uses the synthetic zinc-inducible promoter MRE to switch on transgene expression. LE6 cells were transfected with the ΔMMRE-pNeo expression plasmid using Lipofectamine-plus (Life Technologies, Inc.) and were selected with 400 μg/ml Geneticin (Life Technologies) for 2 weeks. Stable clones were analyzed by Western blot analysis of STAT3 binding (Fig. 6A, B). STAT3 activation by TNF and IL-6. Cells were exposed to IL-6 for 15 min and harvested 1 h later. Lane 1, LE6 cells maintained in 0.5% serum; Lanes 2 and 6, STAT3 binding after TNF stimulation without (Lanes 2) and with TPCK treatment (Lanes 6); Lanes 4, 5, and 7, STAT3 binding after IL-6 stimulation without (Lanes 4 and 5) or with TPCK pretreatment (Lane 7). Supershift analysis of STAT3 binding (Lane 3) was done by incubating 5 μg of protein with 1 μg of STAT3 antibody (Santa Cruz) for 30 min after incubation of the extracts with the SIE probe.

DNA Synthesis Measurements by BrdUrd Incorporation. Growth-arrested LE6 cells were treated with 20 ng/ml of recombinant human TNF for 30 min in the absence of FCS. In some experiments, cells were pretreated with 10 μM TPCK (Sigma) in serum-free medium for 1 h, followed by addition of TNF for the next 30 min. This was replaced for medium containing 0.5% FCS (or 10% FCS in some experiments), and cells were incubated for 14 h. One μg/ml of BrdUrd labeling reagent (Amersham Life Science, Inc.) was added to cultures for an additional 4 h. Cells were rinsed with PBS and fixed with acetic alcohol, and BrdUrd incorporation was measured using the Cell Proliferation kit (Amersham Life Science, Inc.) according to the manufacturer’s instructions. Results are represented as an average percentage of BrdUrd-positive cells counted in three wells. At least 1000 cells were counted in each well. Experiments were repeated at least three times. Cell death was evaluated visually and confirmed by trypan blue staining.

EMSA. Nuclear protein extracts were prepared as described (33). Protein concentration was determined by the Bradford method. The following double-stranded oligonucleotides were used as probes: NF-κB binding sequence from the class I major histocompatibility enhancer element (H2K; DNA International); consensus STAT3 binding oligonucleotides, SIE (cis-inducible element) from the c-fos enhancer, and AP-1 consensus oligonucleotides (the binding site for c-Jun homodimers and Jun-Fos heterodimeric complexes; Santa Cruz Biotechnology; Ref. 5). The NF-κB probes were prepared by end labeling with [γ-32P]ATP using T4 polynucleotide kinase and subsequent column purification. Labeled oligonucleotides were annealed to the complementary oligonucleotides in a thermal cycler in 50 μl Tris (pH 8.0) and 1 μl EDTA. Nuclear protein (5 μg) was incubated with the labeled probe for 30 min at room temperature and electrophoresed through the 5% polyacrylamide Tris-glycine-EDTA gel. Gels were dried and exposed to Kodak X-AR film.

RNA Preparation. RNA was isolated from cells by the guanidine thiocyanate method, followed by ultracentrifugation through cesium chloride as described (34).
PCR reaction was carried out at 94°C for 2 min, 60°C for 1 min, and 74°C for 1.5 min for 36 cycles. Amplified product was electrophoresed through 1.1% formaldehyde-agarose gels and transferred to a nylon membrane (MagnaGraph; Micron Separations). The membrane was prehybridized for 2 h at 42°C and hybridized with the c-myc probe. A portion of cells was preincubated with TPCK for 1 h prior to TNF addition. Cell lysates were prepared 4 h after TNF treatment in caspase lysis buffer, and DEVD-caspase activity was measured as described in “Materials and Methods.” The first three columns represent caspase 3 activity in LE6 cells: column 1, untreated; column 2, exposed to TNF; and column 3, treated with both TPCK and TNF. Columns 4–6, caspase 3 activity in corresponding groups of LE6-9 cells in the presence of 30 μM zinc sulfate. Bars, SD.

**Northern Blot Hybridization.** Ten μg of total RNA were separated by electrophoresis through 1.1% formaldehyde-agarose gels and transferred to a nylon membrane (MagnaGraph; Micron Separations). The membrane was prehybridized for 2 h at 42°C and hybridized with the c-myc probe overnight. A 350-bp PstI fragment from the murine c-myc cDNA, labeled with [γ-32P]deoxyctydine 5′triphosphate by random priming, was used as a probe.

**RT-PCR Analysis of IL-6 mRNA.** The IL-6 mRNA was detected in LE6 cells using reverse transcription-PCR as described previously (5). Briefly, cDNA was synthesized from 1 μg of total RNA using the Gene Amp RNA PCR kit (Perkin-Elmer) in a buffer containing 2 units of murine leukemia virus reverse transcriptase and 2.5 μM oligo(dT) primer. Samples were incubated for 15 min at 42°C, 5 min at 99°C, and 5 min at 5°C. An aliquot representing 500 ng of input RNA was amplified using 0.2 μM rat IL-6 primers (Clontech) and 2.5 units of AmpliTaq polymerase (Perkin-Elmer). PCR reaction was carried out at 94°C for 2 min, 60°C for 1 min, and 74°C for 1.5 min for 36 cycles. Amplified product was electrophoresed through the 2% agarose gel, stained with ethidium bromide, and photographed.

**Western Blot.** Cells were lysed in RIPA buffer [1% NP40, 150 mM NaCl, 50 mM Tris-Cl (pH 7.5), and 0.1% SDS] containing protease inhibitors for 30 min on ice. Cell debris was pelleted at 14,000 rpm in a microcentrifuge at 4°C for 15 min. Protein concentration was measured by the Bradford method. Fifty μg of protein per sample were electrophoresed through the 10% SDS-Tris gel and transferred to a nitrocellulose membrane (Amersham). The membrane was blocked with 5% dry milk in 0.1% TBS-Tween 20 overnight at 4°C and probed with the rabbit IgBα/MAD-3 antibody (Santa Cruz). The horseradish peroxidase-conjugated anti-rabbit IgG (Santa Cruz) was used as a secondary antibody. The ECL detection system was applied for visualization of the specific protein.

**Flow Cytometric Cell Cycle Analysis of LE6 Cells.** Cells were growth arrested for 3 days by maintaining them in medium containing 0.5% FCS. A portion of the cells was treated with TNF for 30 min; the other portion was preincubated with TPCK for 1 h and then treated with TNF. Eighteen h later, cells were trypsinized, washed in PBS, and fixed in 100% ethanol for 1 h at −20°C. Cells were centrifuged, washed with PBS, and stained in 1 ml of 5 μg/ml propidium iodide/RNase in PBS for 30 min at 37°C. Stained cells were analyzed on flow cytometer with the excitation wavelength 488 nm and emission wavelength 590 nm. Multi Plus Software Package (P. S. Rabinovitch; Phoenix Flow Systems, San Diego, CA) was used for processing the data.

**Caspase-3-like Assay.** Cells were incubated with TNF for 4 h and lysed on ice for 10 min in caspase lysis buffer [50 mM Tris (pH 7.4), 1 mM EDTA, 10 mM EGTA, and 10 mM digitonin]. Fifty μg of protein were incubated for 30 min at 37°C in 100 μl of lysis buffer containing 20 μM caspase-3 substrate Ac-DEVD-AMC (Alexis Biochemicals). Fluorescence was measured with an excitation wavelength of 360 nm and an emission wavelength 488 nm and an emission wavelength 590 nm.
wavelength of 460 nm. Substrate autofluorescence was subtracted from each value, and caspase-3-like activity was calculated as relative light units.

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References