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TNF-α Regulates Transforming Growth Factor-α Expression in Regenerating Murine Liver and Isolated Hepatocytes

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TNF-α is a pleotropic proinflammatory cytokine that has been implicated as a contributing factor in a number of disease processes, primarily through its ability to induce the expression of inflammatory and cytotoxic mediators. TNF-α is also involved in cell growth accompanying the healing process in multiple organ systems and influences liver repair following hepatotoxic damage or regeneration following partial hepatectomy. In this respect, TNF-α is a known mitogen for hepatocytes. In this paper we describe a novel role for TNF-α in the modulation of expression of TGF-α, the latter being a complete hepatocyte mitogen. TNF-α directly up-regulates TGF-α mRNA by up to 7-fold in isolated mouse hepatocytes, whereas neutralization of TNF-α significantly decreased liver mRNA and protein expression of TGF-α following chemical-induced hepatotoxicity. That TNF-α directly stimulated TGF-α was suggested by the inability of either anti-IL-6 Abs or cycloheximide to inhibit TNF-α-induced TGF-α expression in hepatocytes. However, in the presence of anti-TGF-α neutralizing Abs, the mitogenic activity of TNF-α is abrogated. Using cells transfected with the TGF-α promoter, and an RNA polymerase inhibitor, it was shown that TNF-α modulates TGF-α expression through both pre- and posttranscriptional events. Taken together, these data suggest that TNF-α participates in liver repair and regeneration, in part, by directly inducing the expression of TGF-α.

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Tumor necrosis factor-α, a 17-kDa polypeptide, was first identified as causing a wasting syndrome in tumor-bearing mice, hemorrhage reduction in the size of some tumors, and necrosis in normal tissues (1–3). Originally identified in a destructive context, TNF-α is now known to provide homeostatic and physiological functions by influencing cell proliferation and differentiation, while causing apoptotic cell death in certain cell types. Due to the influence chronic inflammation plays in disease processes, recent attention has focused on its role in regulating inflammatory processes (1–3) through its ability to regulate genes that code for inflammatory mediators. The mechanisms by which TNF-α can influence physiological processes, such as cell proliferation, have not been well studied but likely involves the mitogen-activating protein kinase pathway leading to activation of transcription factors, such as AP-1, and expression of growth factors.

The liver, unlike most organs, has the capacity to regenerate or repair itself following injury. In experimental animals liver regeneration has been studied using a model of physical injury (i.e., 2/3 partial hepatectomy (PH)2), and liver toxicity by exposure to hepatotoxins such as carbon tetrachloride (CCl4). Although both procedures result ultimately in restored liver mass and function, they differ in that PH is primarily a compensatory hyperplasia, and does not involve an inflammatory response (4), whereas CCl4 can cause injury, necrosis, and a robust inflammatory response that results in far more damage to the organ than the chemical itself (5). Increasing evidence indicates that TNF-α, possibly through its ability to induce IL-6, plays an important role in liver regeneration following PH or CCl4 exposure (6–9). Previous studies by this laboratory (8) and elsewhere (10) have shown that neutralization of TNF-α following CCl4 treatment or PH (6, 11) results in a significant delay in the regenerative process. Furthermore, administration of TNF-α in the rodent increases liver weight (12), induces hepatocyte mitosis, and stimulates liver DNA synthesis (12, 13). In contrast to IL-1 or IL-6 (14, 15), TNF-α is also a mitogen for isolated hepatocytes (14, 15). Additionally, TNF-α will induce IL-6 expression in the liver, and IL-6-deficient mice display a delay in liver regeneration following PH, indicating that IL-6 is also important in regeneration (7).

Cell proliferation is a complex and tightly controlled process, which is modulated by cell-to-cell contact and various growth factors (4). Evidence indicates that TNF-α influences the expression of several of these growth factors. For example, TNF-α can modulate hepatocyte growth factor levels in fibroblast cultures (16) and TGF-α (2) in pancreatic cell lines (17). However, the role of TNF-α in modulating hepatocyte-derived growth factors has not been investigated. TGF-α has been identified as a mitogen for hepatocytes in culture and is one of the major stimuli for cell proliferation during liver regeneration. Although epidermal growth factor and hepatocyte growth factor are produced early in the regenerative process, TGF-α is induced later and is involved in completion of the regenerative process (4). The timing of its induction would make TGF-α a likely candidate for modulation by proinflammatory cytokines that are induced early during liver regeneration. In this paper we describe a novel role for TNF-α in the modulation of expression of TGF-α in hepatocytes. Furthermore, we define the effects of endogenous TNF-α on TGF-α induction following chemically induced hepatotoxicity, thus indicating a distinct role for TNF-α in liver regeneration.

Footnotes:

1 Abbreviations used in this paper: CCl4, carbon tetrachloride; PH, partial hepatectomy; RPA, ribonuclease protection assay; rm, recombinant murine; NBT-BCIP, nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate.

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Materials and Methods

Experimental design

Experimental animals were treated in accordance with the criteria outlined in the “Guide for the Care and Use of Laboratory Animals” (NIH publication 86-23, 1996). Female B6C3F1 (C57BL6 × C3H) mice (The Jackson Laboratory, Bar Harbor, ME), weighing 22–28 g and ~6–8 wk old, were housed in polycarbonate cages containing hardwood chip bedding at room temperature (21 ± 2°C) on a 12-h light/dark cycle. Animals were assigned to groups randomly by weight and administered a single i.p. dose of 0.1 ml/kg CCl₄ (Sigma, St. Louis, MO) or an equal volume of corn oil vehicle as previously described (8). Euthanasia was performed by CO₂ asphyxiation using National Institute for Occupational Safety and Health-approved guidelines for the humane treatment of laboratory rodents. Livers were flash frozen in liquid nitrogen and stored at −70°C until assayed.

Polyclonal antisera to murine TNF-α was prepared and characterized for specificity and titer as previously described (18). Each mouse was injected i.v. with 0.2 ml of prefixed, nonimmune, or immune serum 1 h before CCl₄ administration. This dose effectively neutralizes serum TNF-α activity following endotoxin administration (18).

Hepatocyte isolation

Hepatocytes were isolated from mice and prepared by a modification of the protocol of Seglen (19). Briefly, the liver was retrograde perfused in situ with warmed (37°C) Liver Perfusion Buffer (Life Technologies, Bethesda, MD) followed by Digestion Buffer (Life Technologies). The liver was removed and the cells were gently dispersed into William’s E medium (Life Technologies). Viable cells were enriched by isodensity Percoll (Pharmacia Fine Chemicals, Piscataway, NJ). Aliquots of 1 × 10⁶ cells in 2 ml volumes were seeded into 6-well culture dishes in William’s E medium supplemented with 10% FBS (HyClone, Logan, UT) and 2 mM l-glutamine. The cells were then again allowed to incubate overnight, after which fresh serum-free William’s E medium was added containing 0.2% DMSO with or without treatments. Recombinant murine (rm)IL-6, TNF-α, and anti-IL-6 neutralizing Ab were purchased from R&D Systems (Minneapolis, MN).

Semiquantitative RT-PCR

Cells were collected and total cellular RNA was extracted using the Qiagen RNeasy kit (Valencia, CA) according to the manufacturer’s procedure. cDNA was synthesized as described previously (20). PCR primers for mouse G3PDH and IL-6 were purchased from Clontech (Palo Alto, CA). Primers for mouse TGF-α were custom synthesized (Life Technologies/BRL) from the following sequences: 5’-ACCTGAGGGTTTGGTGCAG and 3’-GGAGGGCGCTGCTTCCTCG (266 bp). Samples of cDNA were amplified by PCR using a GeneAmp PCR System 9600 DNA Thermal cycler. Portions of liver were homogenized with a Dounce homogenizer (10%). The cultures were incubated overnight at 37°C. Following incubation, the medium was replaced with serum-free William’s E medium containing 1 mg/ml BSA and 0.5% DMEM. The cultures were again allowed to incubate overnight. Total RNA was extracted using a single-step nitrocellulose membrane-based protocol (24). The nitrocellulose membrane was incubated with a 1:200 dilution of anti-mouse TGF-α mAb (Santa Cruz Biotechnology, Santa Cruz, CA) for 16 h at 4°C, washed twice, and exposed to goat anti-mouse alkaline phosphatase conjugate (1:500 dilution) according to the manufacturer’s protocol (Santa Cruz Biotechnology). The membrane was developed in 1Step nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT-BCIP; Pierce, Rockford, IL) for ~10–15 min until bands were apparent.

Ribonuclease protection assay (RPA)

Quantification of RNA samples was performed by RPA using the Ribonuclease protection assay (RPA) kit (PharMingen, San Diego, CA) according to the manufacturer’s instructions. The dsDNA template for GAPDH was purchased from PharMingen, and the template for murine TGF-α was generated from a PCR product using mouse TGF-α primers containing a T7 RNA polymerase recognition site. 32P-labeled cRNA probes were produced from dsDNA templates using the MAXiScript T7 kit from Ambion (Austin, TX) according to the manufacturer’s instructions. Aliquots of total RNA were assayed using the Ribonuclease protection assay kit (PharMingen). Samples were electrophoresed on a sequencing gel, and protected fragments were quantitated using a PhosphorImager and ImageQuant software (Storm, Molecular Dynamics, Sunnyvale, CA).

Western blot

Liver cell membrane samples were processed for Western blot analysis essentially as described by Paria et al. (23). Portions of liver were homogenized, centrifuged, and the pellets were resuspended in 10 ml Tris-HCl, 250 mM sucrose buffer containing 10 μg/ml leupeptin, 20 μg/ml PMSF, and 10 μg/ml pepstatin. The total protein concentration was adjusted to 10 mg/ml, and 0.3 mg was separated on a 15% reducing SDS-PAGE gel and electrophoretically transferred to a nitrocellulose membrane as previously described (24). The nitrocellulose membrane was incubated with a 1:200 dilution of anti-mouse TGF-α mAb (Santa Cruz Biotechnology, Santa Cruz, CA) for 16 h at 4°C, washed twice, and exposed to goat anti-mouse alkaline phosphatase conjugate (1:500 dilution) according to the manufacturer’s protocol (Santa Cruz Biotechnology). The membrane was developed in 1Step nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT-BCIP; Pierce, Rockford, IL) for ~10–15 min until bands were apparent. The membrane was rinsed thoroughly in dH₂O and scanned directly with an Eagle Eye II digital imaging system (Stratagene, La Jolla, CA). The digital images were analyzed using NIH Image v1.57.

Transfection of Hepa-1 cells

Hepa-1 cells (American Type Culture Collection, Manassas, VA) were plated at 5 × 10⁵ cells/well in Matrigel-coated 6-well tissue culture plates in DMEM containing 10% FBS, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine (DMEM + 10%). The cultures were incubated overnight at 37°C and 5% CO₂ (70–80% confluent). The TGF-α promoter plasmid (graciously provided by Dr. D.C. Lee, University of North Carolina, Chapel Hill, NC) is a construct of the pGL2 basic vector containing the complete murine TGF-α promoter. An aliquot of purified plasmid construct was incubated with SuperFect reagent (Qiagen) according to the manufacturer’s instructions. The Hepa-1 cells were incubated...
with the plasmid for 2 h, after which the plasmid solution was aspirated and fresh serum-free DMEM containing insulin, transferrin, selenium (ITS; Life Technologies), 100 IU/ml penicillin, 100 g/ml streptomycin, and 2 mM L-glutamine. Treatments were added to individual wells, and the transfected cells were incubated overnight. After incubation, the cells were harvested and samples were prepared for analysis with the Luciferase Reporter Gene Assay kit (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer’s instructions. Luminescence was determined on a Wallac luminescence counter (Wallac, Turku, Finland) and normalized to CellTiter-Glo (Promega). Transfection efficiency was monitored by cotransfection with the pCAT control vector. Chloramphenicol acetyl transferase (CAT) levels were determined from cell-free extracts using the CAT Elisa kit (Boehringer Mannheim) according to the manufacturer’s instructions.

**Statistical analysis**

All experiments were replicated and representative findings are shown. Statistical significance was determined by one-way ANOVA. When the F value was significant, the means were compared using Fisher post hoc analysis. In all statistical comparisons, a p value of <0.05 was used to indicate a significant difference.

**Results**

Initial studies were conducted to determine whether TNF-α could influence the expression of TGF-α in the liver. Hepatocyte isolation can mimic injury (19), and special culture conditions are necessary to maintain basal activity levels following isolation (G. Michalopoulos, unpublished data). Hepatocytes were isolated and cultured on an extracellular matrix (Matrigel) in the presence of low levels of an antioxidant, DMSO. Preliminary experiments found that these conditions reduced the overexpression of growth factors that may occur as a result of liver perfusion and isolation.

Cultured hepatocytes were treated with PMA (20 ng/ml) or various concentrations of rmTNF-α for up to 2 h, and total RNA was isolated from the cells. RT-PCR indicated up to a 7-fold relative increase in TGF-α mRNA occurred following 10 ng/ml TNF-α treatment, which was comparable to that induced by PMA (Fig. 1). When kinetic studies were performed, it was found that maximum induction of TGF-α by TNF-α occurred after 1–3 h of culture (data not shown). Because RT-PCR is a semiquantitative procedure, and can be influenced by factors such as pipetting error, or cycling beyond the linear amplification range of the reaction, RPA was performed on mRNA from TNF-α-treated hepatocytes. RPA is a quantitative measure for specific mRNA. These studies indicated that the increase in TGF-α expression was >3-fold (Fig. 2).

Accumulating evidence has suggested that liver regeneration following either PH (11) or CCl₄ administration (9) is due to the ability of TNF-α to stimulate IL-6 secretion, and that IL-6 may represent the proximal mediator for hepatocyte growth. To explore the possibility that IL-6 is responsible for TGF-α induction, TNF-α, IL-6, or Abs to IL-6 were added to hepatocyte cultures and the expression of TGF-α monitored. According to the manufacturer, the 50% neutralization dose (ND₅₀) for the lot of anti-IL-6 neutralizing Ab used was ~0.001–0.003 μg/ml in the presence of 0.25 ng/ml rmIL-6 (R&D Systems anti-mouse IL-6 Ab, lot number AHV01). To ensure that sufficient anti-IL-6 Ab was present to neutralize any IL-6 produced, ~5000-fold excess of the ND₅₀ was added. TNF-α was capable of enhancing TGF-α expression when added to isolated mouse hepatocytes, and concomitant addition of IL-6 neutralizing Abs did not alter TGF-α expression (Fig. 3). Consistent with these observations, similar concentrations of IL-6 failed to influence TGF-α expression. To determine whether de
 novo protein synthesis was required for the induction of TGF-α message by TNF-α, hepatocytes were stimulated with TNF-α in the presence of the protein inhibitor, cycloheximide, and TGF-α message examined by RT-PCR. As shown in Fig. 4, the ability of TNF-α to stimulate TGF-α message was similar in the presence or absence of cycloheximide, indicating that TNF-α can directly induce TGF-α. Addition of cycloheximide alone had no effect on TGF-α expression (data not shown).

TNF-α is a potent in vivo and in vitro hepatocyte mitogen (14, 25, 26). To help determine whether its mitogenic activity is ultimately due to TGF-α production, TNF-α-stimulated hepatocytes were cultured in the presence of Abs to TGF-α and proliferation was monitored by [3H]TdR incorporation (Fig. 5). The increase in thymidine incorporation induced by TNF-α was ~4-fold above medium control. However, the addition of anti-TGF-α Abs to the culture medium completely abolished the increase in DNA synthesis, suggesting that the stimulatory effect of TNF-α is primarily due to its ability to stimulate TGF-α.

Increases in mRNA levels may result from enhanced transcriptional activity or accumulation of mRNA due to altered degradation. To assess the contribution of mRNA stability to the increase in TGF-α mRNA, Hepa-1 cells were treated with TNF-α followed by actinomycin D, and TNF-α and TGF-α mRNA degradation was evaluated as a function of time using the RPA. As previously established (27), the production and degradation of TNF-α message occurs rapidly after stimulation (Fig. 6). In contrast to TNF-α, the degradation of TGF-α message, following identical stimulation, proceeded considerably slower over the 2-h period, suggesting that stabilization may contribute to the abundance of TGF-α mRNA (Fig. 6). To determine whether TNF-α could also influence TGF-α transcriptional activity, Hepa-1 cells were transiently transfected with the full-length TGF-α mouse promoter linked to a luciferase reporter gene. Transfected cells treated with TNF-α displayed a 3-fold increase in luciferase activity compared with vehicle controls, which was similar to that observed with the positive control, PMA (Fig. 7). Taken together, these data suggested that elevated levels of TGF-α message are due to both transcriptional activation and mRNA stabilization.

To determine whether TNF-α can modulate TGF-α in vivo, mice were administered TNF-α or IL-6 at concentrations known to induce an acute phase response in the liver (28), and the expression of TGF-α was monitored by RT-PCR (Fig. 8). Two hours following TNF-α treatment, a 6-fold relative increase in TGF-α expression was observed in the liver, while administration of rmIL-6 had no effect on its expression.

To further characterize the role of TNF-α in chemically induced hepatotoxicity, mice were administered an acute dose of CCl₄ (0.1 ml/kg), a concentration which has been shown to cause temporary liver damage and TNF-dependent repair (8), and livers were examined by RT-PCR for relative changes in the expression of TGF-α, and IL-6. We (8) and others (29) have previously demonstrated that CCl₄ treatment induced robust TNF-α expression in the liver. In addition to TNF-α, CCl₄ treatment had an pronounced effect on the mRNA expression of IL-6, and TGF-α (Fig. 9) with
the latter increased maximally (3-fold) within 12–24 h after CCl₄ exposure. Furthermore, neutralizing Abs to TNF-α administered before CCl₄ exposure fully prevented the induction of IL-6 and TGF-α. These observations were verified by RPA, which also indicated that CCl₄ induced a 2- to 3-fold increase in TGF-α expression (Fig. 10), which could be prevented by neutralizing Abs to TNF-α. To determine whether the increase in TGF-α gene expression was associated with altered protein levels, membrane bound pro-TGF-α levels were determined by Western blot analysis. Fig. 11 shows that CCl₄ exposure resulted in a significant increase in pro-TGF-α protein that was attenuated by pre-treatment with neutralizing Abs to TNF-α.

Discussion

The importance of TNF-α as a regulator of liver regeneration and repair was originally demonstrated in rats in which administration of neutralizing Abs to TNF-α before PH impaired liver regeneration (6). TNF-α neutralization also prevented the activation of “early immediate genes,” such as those regulated by the transcription factors NF-κB and AP-1, which are involved in cell proliferation (7, 10). This is consistent with the reported ability of TNF-α to increase hepatic DNA and RNA synthesis and hepatic mitosis (14). Repair following CCl₄-induced damage occurs through similar processes (8, 10). However, the former is characterized initially by marked inflammation and necrosis, where TNF-α has a
suspected role (4). CCl₄ is metabolized to the highly reactive chloro-trimethyl radical which interacts with membrane lipids and initiates lipid peroxidation (30). Secondary liver injury following CCl₄ exposure may occur from inflammatory processes originating from products of activated Kupffer cells such as TNF-α. TNF-α can induce the production and release of chemoattractants and activators of neutrophils. The resulting neutrophil influx promotes extensive tissue damage, including fibrosis, via the release of reactive nitrogen and oxygen species (31).

To date, studies on the role of TNF-α in liver repair have focused almost exclusively on changes in early-immediate genes. In the present studies we focused on upstream events, specifically the ability to modulate the complete hepatocyte mitogen TGF-α. TGF-α, which is mitogenic for hepatocytes and other liver-derived cells, is thought to be a major stimulus for liver regeneration and has been linked to the proliferative response in the liver following PH (32). TGF-α is expressed as a 160-aa membrane-integral precursor and a 50-aa mature form. The mature form is only detectable after hepatocyte proliferation commences, indicating that the membrane-bound form is responsible for the majority of the activity during regeneration (33). Using isolated murine hepatocytes as a model, as well as chemically induced hepatotoxicity, we demonstrated that TNF-α can directly stimulate TGF-α expression in hepatocytes. The relative increase in hepatic TGF-α expression observed in the present studies was only 3- to 4-fold following in vivo TNF-α administration, which is similar to the increase observed after PH (33). It is thought that the low induction rate is due in part to the fact that TGF-α elicits responses in adjacent cells in a juxtacrine fashion, primarily through membrane-bound TGF-α rather than the soluble form. Several hours following liver damage, TGF-α mRNA levels increase, peaking at approximately the same time as the first wave of DNA synthesis (33).

As indicated earlier, increasing evidence has suggested that the signaling pathway by which TNF-α regulates liver regeneration is through activation of the downstream events involving sequential activation of NF-κB, IL-6 secretion, and eventually STAT3 activation. The importance of IL-6 in liver regeneration was demonstrated by the ability of IL-6 injections to correct the deficiency in hepatic DNA synthesis and restore STAT3 binding to normal levels in PH or CCl₄-treated TNF receptor or IL-6-deficient mice (10, 11). As we observed that hepatic IL-6 mRNA was increased following CCl₄ administration and reversed in animals pretreated with neutralizing TNF-α Abs, it could be assumed that IL-6, rather than TNF-α, was also the proximal mediator of TGF-α induction. However, injection of IL-6, even at concentrations as high as 1 mg/kg, failed to influence TGF-α expression, whereas injection of TNF-α induced a robust response. These data are consistent with a recent study, which demonstrated that IL-6 injection before CCl₄ treatment inhibited DNA synthesis in livers of wild-type mice (10). In vitro studies further demonstrated that IL-6 did not induce proliferation or TGF-α expression in isolated hepatocytes from normal mice. Furthermore, cycloheximide did not affect the ability of TNF-α to induce TGF-α, indicating that de novo protein synthesis was not a prerequisite. These findings attest...
to the fact that liver regeneration represents a complex process with numerous redundancies, which can be exemplified by the observation that liver regeneration in TGF-α null mice occurs as rapidly as in wild-type mice following PH (34). This is presumably due to compensation by epidermal growth factor. Furthermore, it should be noted that liver regeneration following CCl₄ exposure is only delayed in the absence of TNF-α, suggesting that other direct acting hepatocyte mitogens can compensate.

Multiple NF-κB and an AP-1, but no STAT-3, binding sites exist in the promoter region of the TGF-α gene (35). Both NF-κB and AP-1 activation are associated with TNF-α-mediated signaling events in the liver (6), and administration of TNF-α increases their DNA binding activities in hepatocytes as demonstrated by EMSA (36). Furthermore, hepatocyte proliferation following CCl₄ exposure is preceded by NF-κB (9) and AP-1 (8) activation which can be prevented or significantly delayed by TNF-α neutralization. The murine TGF-α gene also contains several poly(A) U sites in the 3’-untranslated region that have been postulated to modulate the stability of the transcript (37). Message stabilization and cell transfection studies provided evidence that the increase in TGF-α message was due to both increased synthesis and message stabilization. Consistent with our observations, it has been postulated that the initial increase in hepatic mRNA following PH is mediated by transcriptional activation, and within 3 h after PH, gene regulation is switched primarily to posttranscriptional mechanisms (38).

Although the mechanisms by which TNF-α regulates liver repair are likely quite complex, the current studies suggest that TNF-α-induced TGF-α expression is involved by acting upstream. There is little doubt that other processes, including those controlled by the IL-6 and the CCAAT/enhancer-binding protein transcription factor families, also participate. Current studies are focusing on the specific molecular mechanisms that control these events.

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