TNF-α modulates expression of the tissue transglutaminase gene in liver cells

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THE CALCIUM-DEPENDENT specific cross-linking of ε-amines and γ-glutamyl residues is accomplished by a family of enzymes termed transglutaminases (1, 9, 10). These enzymes participate in a variety of cellular and tissue functions and have been implicated in the formation of stabilizing cross-links among extracellular protein components. For example, a distinct keratinocyte transglutaminase is upregulated during epidermis formation and catalyzes significant cross-linking among keratin and several additional extracellular proteins (12). Furthermore, the establishment of fibrin clots is dependent on activation of factor XIIIa, another transglutaminase (15). An additional family member, the ubiquitous tissue transglutaminase (tTG), can be localized to the cell membrane and detected in a secreted form, and tTG appears to be present intracellularly, partitioning between membrane-bound and soluble compartments (1). The physiological function of this intracellular form remains largely unknown. However, it has been known for some time that tTG also has guanosine triphosphatase activity as well, and recent studies identify tTG as the GTP-binding, G subunit, which couples the αβ-adrenergic receptor to a unique form of phospholipase C (19). Such data implicate tTG in a variety of signal transduction events in addition to cross-linking activity.

Several groups have described a role for tTG in cross-linking fibronectin, osteonectin, osteopontin, laminin, nidogen, and other extracellular matrix components (1, 9). Analogous to its noted participation in wound healing, it is tempting to speculate that extracellular tTG may participate in other tissue remodeling processes subsequent to cell injury and death, such as fibrogenesis. Indeed, we recently documented a dramatic rise in tTG activity during the induction of liver fibrosis in rats by CCl4 damage and in human patients with acute liver disease (17).

Liver fibrosis represents a common terminal stage of disease precipitated by a variety of etiologies leading to sustained cellular injury. The initiation of fibrogenesis appears to involve several events mediated by proinflammatory and cytotoxic cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1 and IL-6, and transforming growth factor (TGF)–β isomers (5, 7). It would therefore be reasonable to suggest that tTG could participate in the deposition of excess extracellular matrix seen in fibrotic diseases. Because our earlier studies also revealed an increase in nuclear factor (NF)-κB binding to tTG promoter sequences accompanying liver fibrosis and TNF-α is known to be a major activator of NF-κB binding, a potential role for TNF-α is strongly suggested in tTG expression. The following in vitro experiments support a significant role for TNF-α modulation of tTG gene activity.

MATERIALS AND METHODS

Cell culture and manipulation. Cultures of human hepatoblastoma cells (Hep G2) or cervical carcinoma cells (HeLa) were incubated at 37°C in 5% CO2 in modified Eagle's medium supplemented with 100 U/ml penicillin, 100 U/ml
streptomycin, and 10% fetal calf serum (Life Technologies, Gaithersburg, MD). Cells were inoculated into 10 ml of medium in 100-mm plastic petri dishes (Corning, NY) or into 3-ml medium per well in six-well culture plates and incubated until 70–80% confluency was achieved. We noted that addition of large amounts of serum-containing medium stimulated an increase in transglutaminase activity. As a result, no more than 20 ml of fresh medium with or without 1 ng/ml (440 U) human TNF-α (Genzyme, Boston, MA) per milliliter of culture medium were added for a period of 1–24 h before cell harvest. This protocol placed cells at the final stages of exponential growth at the time of assay.

For some experiments we used cells from lineage 27, a mouse line transgenic for the β-galactosidase reporter driven by 4 kb of the murine tTG promoter (L. Nagy, V. A. Thomazy, M. M. Saydak, J. P. Stein, and P. J. A. Davies, unpublished data). Primary mouse hepatocytes, grown in six-well cultures containing William's E medium supplemented with 10% fetal calf serum, were isolated as previously described (17). TNF-α treatment was as previously described.

Assay for transglutaminase activity. Cells were inoculated into six-well plates and grown as previously described. TNF-α at 1 ng/ml was added to the medium at 1, 2, 6, or 24 h before terminating the experiment. One hour before cell harvest, 250 µl of [14C]putrescine (100 µCi) was added to each culture. Cells were washed once with ice-cold Hanks' buffered salts, scraped into centrifuge tubes, and pelleted by low-speed centrifugation. The cell pellet was washed once more with Hanks' salts and transferred to a 1.5-ml microfuge tube. After low-speed centrifugation the cell pellet was precipitated with 5% trichloroacetic acid (TCA) and placed on ice for 15 min. The insoluble protein pellet was washed two times with ice-cold 5% TCA and recovered by high-speed centrifugation. After solubilization of the pellet in 1 N NaOH, radioactivity was determined by liquid scintillation counting. Samples were normalized to protein by bicinchoninic acid (BCA; Ref. 24) determination of an aliquot of each solubilized pellet.

Plasmid transfection and stable line construction. Equimolar amounts corresponding to 3 µg of the pHTGP2 or pHTGP2-mut3 (16) constructs were used in all calcium-phosphate transfections (20). The plasmid DNA was mixed thoroughly in 220 µl of sterile H2O in a 15-ml conical tubes. Sterile 2 M CaCl2 (30 µl) was added slowly to the surface of the DNA solution followed by the slow addition of 250 µl of sterile 2× Hanks' buffered salt solution (in mM: 280 NaCl, 10 KCl, 1.5 Na2HPO4, 12 glucose, 50N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; pH 7.05) to the bottom of the tube. The solution was then mixed gently and a DNA-calcium-phosphate precipitate was allowed to form at room temperature. The precipitate was mixed and gently added to a 10-ml 100-mm petri dish cell culture. The cultures were subsequently incubated overnight for 16–20 h. The medium was removed, and the cells were recovered by trypsinization and low-speed centrifugation. The cells were then repelleted into wells of a six-well plate, and incubation was continued for 24 h to allow the cells to recover. Treatment with TNF-α began at this time. Control experiments previously demonstrated that reporter expression continues to be elevated during this period. Untreated, paired time controls were comparable, indicating that expression of the transiently transfected reporter was equivalent over the course of the experiment.

A stable Hep G2 cell line was produced by cotransfecting 10 µg of the mutant p50 expression construct, cytomegalovirus (CMV)-ΔSP, a kind gift of A. Israel (14), with 1 µg of a plasmid containing the neomycin-coding region driven by the SV40 promoter and enhancer (25). After 24 h the cells were trypsinized, and neomycin-resistant cells were selected by replating in medium containing 700 µg/ml of the neomycin analog G-418. Incubation continued for 3 wk with frequent replacement of selection medium. Several clones were selected and expanded, and frozen stocks were prepared. Experiments were performed on a clonal stock that had previously tested as having no detectable nuclear extract binding to NF-κB DNA motifs after 24 h of treatment with TNF-α.

Lines of HeLa cells stably transfected with either the full-length tTG promoter pHTGP2 or the NF-κB-deficient promoter pHTGP2-mut3 were generated as above.

Luciferase and β-galactosidase expression. After incubation the cultures were washed once in phosphate-buffered saline. Cells were lysed by the addition of 200 µl of cell lysis buffer (Promega, Madison, WI) followed by gentle shaking. The lysate was transferred to a 1.5-ml tube and clarified with a 2-min centrifugation at 16,000 rpm. The pellets were discarded, and the supernatants were either assayed immediately or stored at −70°C. The protein concentration of the lysate was determined by the BCA method of Smith et al. (24).

Luciferase activity was assayed, with modifications, according to deWet et al. (4). Reactions were performed in 300-µl volumes containing 25–200 µl of cell lysate and reaction buffer at a final concentration of 0.125 M tris(hydroxymethyl)aminomethane (pH 7.2) and (in mM) 1 dithiothreitol, 1 ATP, 0.5 acetyl CoA, and 5 MgCl2. Light emissions were measured directly and integrated over a 30-s period at room temperature after the injection of 100 µl of freshly prepared α-luciferin (Sigma Chemical, St. Louis, MO) into a luminometer (Lumat LB9501, Berthold Analytical, Nashua, NH). Blank reactions were determined with equivalent volumes of lysis buffer substituted for cell lysates.

The data were expressed as relative activity calculated as the luciferase activity per milligram protein of the test construct divided by the luciferase activity per milligram protein of the untreated samples. β-Galactosidase was measured on cell lysates as per the manufacturer's recommendations (Promega, Madison, WI) and normalized to untreated control cell lysates.

mRNA analysis and electrophoretic mobility studies. Northern analysis for tTG transcripts was performed as previously described (17) on RNA extracted from Hep G2 cultures grown in T-25 flasks with 10 ml medium. Cultures were incubated with no or 1 ng/ml TNF-α for 1, 2, 6, or 24 h before extraction. Gel-mobility shift analyses were performed using nuclear extracts from untreated or 1-, 6-, or 24-h TNF-α-treated Hep G2 cultures grown in T-25 flasks as previously described (17).

RESULTS

Using the active incorporation of the externally applied amine donor [14C]putrescine into Hep G2 cellular proteins as an assay for transglutaminase, we noted a time-dependent increase in incorporation after treatment with low levels of TNF-α (Fig. 1). It can be seen that activity is elevated after only 1 h of treatment of TNF-α and that maximal activity is seen at 6 h. Activity is still increased after a 24-h incubation with the cytokine. The increase in transglutaminase activity is
not correlated to Hep G2 cell death because previous studies indicated that at the levels of TNF-α used in these experiments there is no cell necrosis (21).

Because of complex regulation, the cross-linking activity of tTG can be modulated without prior synthesis of protein (3, 23). Therefore, enzyme levels cannot be used as a direct assay of mRNA levels or transcriptional activity. We directly examined changes in the levels of mRNA specific for tTG via Northern blot analysis after treatment with TNF-α. The results depicted in Fig. 2 show good correlation with the previously determined enzyme levels. Again, mRNA levels (normalized to glyceraldehyde-3-phosphate dehydrogenase levels) increase at 1 h to a maximum at 6 h, remaining high after 24 h of incubation with TNF-α.

Using subconfluent cultures of Hep G2 cells for transfection, a luciferase construct containing 1.67 kb of the human tTG promoter (pHTGP2, nucleotide position +72 to −1,665), and an assay of luciferase activity as a reporter for tTG promoter response, we observed (Fig. 3) that treatment with TNF-α stimulated the expression of luciferase activity. The time course and magnitude of this response were almost identical to that observed with the mRNA levels noted in Fig. 2. Because of the transfection experimental protocol used with these experiments, expression level data are normalized to untreated culture controls, thus avoiding the need for transfection efficiency measurements. In contrast, deletion of 1.1 kb of upstream sequences in the tTG promoter (pHTGP2-mut3, nucleotide position +72 to −561) results in a highly attenuated response to TNF-α (Fig. 3). The truncated 0.56-kb construct, pHTGP2-mut3, was previously shown to have no decrease in constitutive activity under normal conditions compared with the full length 1.67-kb promoter construct pHTGP2 (16). The deleted region was noted to contain several other transcription regulatory binding motifs, including a glucocorticoid response element at −1,399 and an IL-6 response element at −1,190, in addition to the putative NF-κB binding motif at −1,338. Therefore, sequences within the deleted upstream segment must be involved in the appropriate upregulation in response to TNF-α.

To determine whether the effects we observed in the hepatoma-derived cell line also occurred in other cells, we undertook studies with HeLa cells. HeLa cells
stably transfected with the 1.67-kb tTG reporter construct also showed stimulation of activity after incubation for 24 h with TNF-α (Fig. 4). Moreover, HeLa cells stably transfected with the truncated, 0.56-kb tTG reporter construct showed an attenuated response to TNF-α, as had occurred in the Hep G2 system. In addition, freshly isolated primary hepatocytes from a transgenic mouse line, stably transfected with a 4-kb tTG promoter-β-galactosidase reporter construct, showed stimulation of reporter activity after incubation for up to 24 h with TNF-α (Fig. 5).

With use of a segment of the tTG promoter containing the NF-κB binding motif and nuclear extracts from untreated, control Hep G2 cells or Hep G2 cells treated for varying times with TNF-α, gel-mobility shift analyses were performed. A demonstrable increase in binding of treated culture nuclear extracts to the NF-κB binding motif could be seen compared with untreated, control extracts (Fig. 6). The rise in binding activity was seen at 1 h of cytokine incubation, and like the protein, mRNA, and reporter levels, increased at 6 h followed by a slight decrease (but still elevated level) after 24 h of incubation. Control experiments with cold excess oligonucleotides and with p50 and p65 antibodies confirm the specificity of the binding reaction and identification of the p50 subunit as one of the bound NF-κB species (data not shown). These findings therefore strongly point to a role of NF-κB binding to the tTG promoter and upregulating gene activity in response to TNF-α treatment. To test this hypothesis further, we stably transfected Hep G2 cells with a plasmid expressing a nonfunctional, transdominant mutant of the p50 NF-κB subunit. Control experiments show little binding of nuclear extracts from these cells to consensus NF-κB sequences even after incubation for 24 h with TNF-α (data not shown). In contrast to cells lacking this mutant, transient transfection of these cells with a full length, 1.67-kb promoter construct shows no increase in activity with TNF-α treatment (Fig. 7).

**DISCUSSION**

We have been interested in the mechanisms by which fibrogenesis proceeds in the liver after injury. Extensive fibrosis is a common pathway in many progressive liver diseases, and inflammatory events typically produce hepatocyte damage. Several cytokines are known to be secreted by resident liver cells, as well as invading immune monocytes, T cells, or B cells. Among these cytokines are TNF-α, IL-1β, the TGF-β isoforms, and IL-6, whose effects on hepatic tissue during the injury process are well studied (5, 7). Consequently, this cascade of inflammatory mediators and their paracrine, juxtacrine, or autocrine effects, when sustained, often leads to excess extracellular matrix deposition and the architectural disruption of hepatic tissue and function.

We have become intrigued by the expression of the enzyme tTG, whose role in cross-linking extracellular matrix proteins is well documented (1). Speculative roles of such cross-linking in wound healing (27) and liver damage (11, 22) have been put forth. In addition, tTG activity in hepatoma cell lines is stimulated by cytokines such as TGF-β (8) and IL-6 (26). These observations suggest a possible involvement of transglutaminase activity in the generation of stabilizing, cross-linked extracellular matrix molecules during tissue assembly and maintenance. In particular, increased
activity could be regulated by known matrix-promoting mediators of injury and inflammation. Because TNF-α is known to be expressed in hepatic injury, regeneration, and fibrosis and because it enhances NF-κB binding to a variety of genes (5, 6), it seemed reasonable to investigate the role of this cytokine in affecting changes in the activity of the tTG gene via binding of NF-κB.

This hypothetical role is supported by our previous studies, in which liver fibrosis was induced by prolonged exposure to CCl₄ (17). Those data show a rapid rise in transglutaminase activity in all hepatic cell types. The rise in enzyme activity is correlated with an increase in tTG mRNA activity as well. When tTG mRNA levels were measured in normal human versus fulminant liver disease, a large increase was seen in the diseased livers. After a computer search of potential transcriptional motifs in the tTG promoter, a putative NF-κB site (~1.3 kb upstream from the start site) was identified. Binding of nuclear extracts to this region increases in parallel to tTG mRNA activity during the course of induced chronic liver disease.

To investigate the regulation of tTG expression in more detail we turned to an in vitro model of hepatocyte response, the Hep G2 hepatoblastoma cell line. We show by analysis of enzymatic activity, mRNA levels, and promoter activation that TNF-α produces a modest, but significant, stimulation in all cases. Furthermore, 1) specific nuclear factor binding activity to a TG NF-κB promoter motif is enhanced by TNF-α treatment, 2) deletion of this motif in the tTG promoter regions leads to a loss of this TNF-α response, and 3) expression of a promoter reporter is diminished in cells with an attenuated NF-κB binding capacity. The data therefore implicate TNF-α in upmodulating the activity of the tTG promoter. This response appears to be generalizable because data obtained with HeLa cells corroborate the results observed with Hep G2 cells. Similarly, the tTG promoter in primary murine hepatocytes appears to respond to TNF-α treatment in an identical fashion. Moreover, glial transglutaminases are elevated in the presence of TNF-α (18).

Our results indicate a sustained increase in NF-κB binding over the course of incubation with TNF-α. This phenomenon may be explained by the subsequent activation of both TNF-α and IL-6 genes in cells by NF-κB. This could result in a sustained autocrine stimulation of the tTG promoter at both the NF-κB promoter motif and its neighboring IL-6 response element. The deleted region in the truncated reporter construct contains several transcription regulatory binding motifs, including a glucocorticoid response element at −1,399 and the IL-6 response element at −1,190, in addition to the putative NF-κB binding motif at −1,338.

It is therefore possible that these elements could additionally modulate the level of tTG gene expression during exposure of cells to TNF-α. In other experiments (data not shown) we have also demonstrated elevation of tTG promoter activity by either TGF-β or IL-6 after

Fig. 6. Electrophoretic mobility analysis of Hep G2 nuclear extracts and the tTG promoter NF-κB sequence motif. Nuclear extracts were prepared from Hep G2 cells treated for varying periods with TNF-α. Gel-mobility shift analyses were performed after incubation of nuclear extracts with a [³²P]-labeled, double-stranded oligonucleotide containing the NF-κB binding motif of the human tTG promoter. Lane 1, untreated cells; lane 2, cells treated for 1 h with TNF-α; lane 3, cells treated for 6 h; lane 4, cells treated for 24 h. This assay is representative of 3 separate experiments.

Fig. 7. Promoter analysis of Hep G2 cells containing mutated NF-κB subunit p50. Hep G2 cells were stably transfected with a constitutively expressing mutant p50 construct. No NF-κB DNA binding could be detected with nuclear extracts prepared from these cells. The stable cell line was calcium phosphate transfected with a 1.67-kb human tTG promoter-luciferase construct. After transient transfection, cells were replated and treated for varying durations with TNF-α. Luciferase and protein assays were performed on cell lysates. Data are presented as the ratio of treated light units per milligram protein to untreated light units per milligram protein. Each point represents triplicate samples with SD error bars.
incubation for 24 h. Therefore, our data may reflect a bipartite mode of regulation with a TNF-\(\alpha\) role in early events, followed by prolonged increases involving other factors. We conclude that hepatocytes are susceptible to rapid stimulation of the TG gene by TNF-\(\alpha\), a cytokine that is upregulated during the fibrogenic process. Chronic production of TNF-\(\alpha\) and other inflammatory mediators could stimulate the synthesis and release of this enzyme, which in turn could play a significant role in the initiation, formation, and sustained levels of the disruptive extracellular matrix seen in many fibrotic diseases. Control of transglutaminase activity therefore may result in important and innovative therapies for this significant disease.

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