TNF-α inhibits liver collagen-α₁(I) gene expression through a tissue-specific regulatory region

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Houglum, Karl, Martina Buck, Dong Joon Kim, and Mario Chojkier. TNF-α inhibits liver collagen-α₁(I) gene expression through a tissue-specific regulatory region. Am. J. Physiol. 274 (Gastrointest. Liver Physiol. 37): G840–G847, 1998.—Although tumor necrosis factor-α (TNF-α) inhibits collagen-α₁(I) gene expression in cultured hepatic stellate cells, assessment of its effects on hepatic collagen expression is complicated by the confounding variables of tissue necrosis and inflammation. Therefore, we analyzed whether chronically elevated serum TNF-α affects constitutive hepatic collagen gene expression in vivo by inoculating nude mice with Chinese hamster ovary (CHO) cells secreting TNF-α (TNF-α mice) or with control CHO cells (control mice). Before the onset of weight loss, collagen synthesis and collagen gene expression were inhibited in the liver of TNF-α mice. In transgenic mice, after 8 h, TNF-α (500 ng at 0 and 5 h) inhibited the liver expression of the collagen-α₁(I)-human growth hormone (hGH) transgene containing the first intron and −440 bp of the 5′ region. Similarly, in cultured hepatic stellate cells isolated from these transgenic animals, the −440 bp collagen-α₁(I)-hGH transgene was responsive to TNF-α treatment independent of the activation of these cells. Transfection studies in stellate cells allowed further characterization of this TNF-α-responsive segment to −220 bp of the 5′ region. Because in the skin the inhibitory effect of TNF-α involves a regulatory region of the collagen-α₁(I) gene beyond −440 bp, we herein identify a novel tissue-specific regulation of collagen-α₁(I) gene by TNF-α.

METHODS

Mouse model of cachexia. Chinese hamster ovary (CHO) cells were stably transfected with either the human TNF-α gene cloned into a mammalian expression vector (TNF-α cells) or the mammalian expression vector alone (CHO cells, control) as described previously (5, 7, 8). Cells were grown in DMEM supplemented with 10% FCS, penicillin (50 U/ml), and streptomycin (50 µg/ml). The TNF-α cells, but not the CHO cells, produced TNF-α as measured by either cytolytic or ELISA assays. Four-week-old male nude mice were injected intramuscularly with either 10² CHO cells or 10³ TNF-α cells as described previously (5, 7, 8). Nude mice were housed in a temperature- and humidity- controlled facility. Animals had free access to food and water, and they were killed at 14–21 days (before the onset of weight loss of the TNF-α mice). Serum TNF-α levels were determined by an immunoassay using monoclonal antibodies against human TNF-α (Endogen) (5, 7). In other experiments, adult C57BL/6 mice were injected intraperitoneally with either 500 ng of human recombinant TNF-α or saline (control) at 0 and 5 h and killed at 8 h. At least three animals were used for each group in all experiments.

Determination of specific activities of liver collagen and noncollagen proteins. Mice were fed ad libitum and had free access to water. Animals were given intraperitoneal injections of 200 µCi of [5-³H]proline in 0.5 ml of sterile saline, and after 2 h they were killed and the liver was removed and placed immediately in ice-cold buffer A (10, 12). This buffer contains 50 mM Tris-HCl, 15 mM EDTA, 1 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, and 5 mM N-ethylmaleimide, pH 7.5. The liver was homogenized for 1 min at speed setting 7 of a DuPont Sorval Omni-Mixer. Portions of liver homogenates were used to determine the specific activity of liver proteins, as described below. The remainder was used to purify collagen as described previously (10). After centrifugation of liver homogenates at 3,000 g for 30 min at 4°C, the precipitates were suspended in 0.5 N acetic acid containing analysis of constitutive collagen gene expression. Therefore, we analyzed the role of TNF-α on hepatic stellate cell collagen gene expression independent of hepatic necroinflammation or stellate cell activation.

We found that both chronic and short-term administration of TNF-α inhibit constitutive liver collagen-α₁(I) gene expression. In addition, we established that in the liver or in cultured hepatic stellate cells isolated from transgenic mice, the −440 bp 5′ segment and the first intron of the collagen-α₁(I) gene were sufficient for responsiveness to TNF-α treatment. Furthermore, transfection of chimeric reporter genes to hepatic stellate cells allowed us to circumscribe the TNF-α responsiveness to the −220 bp 5′ region. Because in the skin the inhibitory effect of TNF-α involves a regulatory region of the collagen-α₁(I) gene beyond −440 bp (8), we herein identify a novel tissue-specific regulation of collagen-α₁(I) gene by TNF-α.
pepsin (10 mg/g) and were incubated for 6 h at 4°C with stirring. The suspensions were centrifuged at 3,000 g for 30 min at 4°C; the supernatant solutions were adjusted to pH 7.5 with 2 N NaOH. The pepsin digestion was repeated twice. The supernatants were combined and precipitated with 176 mg/ml (NH4)2SO4, pH 8, and the precipitates were collected by centrifugation at 14,000 g at 4°C for 1 h. A second (NH4)2SO4 precipitation of pepsin-purified collagen was carried out and the precipitates were washed with 10 ml 70% ethanol and dissolved in 0.1 N NaOH. The amount of collagen was determined by the absorbance at 562 nm produced with the biocinchonic acid (BCA) colorimetric assay (sample: reagent, 1:20; 50°C for 40 min) using calf skin collagen as a standard. Portions of the NaOH solutions were also used to measure the radioactivity. The liver collagen specific activity was calculated from the radioactivity incorporated into collagen and expressed as dpm/mg of collagen.

Liver proteins were precipitated from liver homogenates three times with 66% ethanol, and the precipitate was collected at 3,000 g at 4°C for 10 min. The precipitate was then solubilized with 0.2 N NaOH and stored at −20°C. The specific activity of the total liver proteins (dpm/mg) was determined by the amount of protein measured with the BCA specific activity of the total liver proteins (dpm/mg) was then solubilized with 0.2 N NaOH and stored at 20°C. The specific activity of the total liver proteins (dpm/mg) was determined by the amount of protein measured with the BCA colorimetric assay, using albumin as standard, and the radioactivity quantified by liquid scintillation spectroscopy.

SDS-PAGE. SDS-PAGE was performed as described previously (10). An LKB vertical slab gel was employed with SDS-PAGE. The liver collagen specific activity was calculated from the radioactivity incorporated into collagen and expressed as dpm/mg of collagen.

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Table 1. Analysis of collagen production in CHO and TNF-α mice

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Liver Protein Specific Activity, dpm/μg</th>
<th>Relative Rate of Collagen Production, %</th>
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</thead>
<tbody>
<tr>
<td>CHO</td>
<td>Total protein</td>
<td>10.8</td>
</tr>
<tr>
<td></td>
<td>Collagen</td>
<td>9.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12.0</td>
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<tr>
<td>Mean ± SE</td>
<td></td>
<td>529 ± 49</td>
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<tr>
<td>TNF-α</td>
<td></td>
<td>454</td>
</tr>
<tr>
<td></td>
<td></td>
<td>624</td>
</tr>
<tr>
<td></td>
<td></td>
<td>464</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td></td>
<td>554 ± 80</td>
</tr>
</tbody>
</table>

Purified liver collagen specific activity was expressed as percentage of total liver protein specific activity after correction for 5.4-fold higher content of proline (+hydroxyproline) in collagen (12). TNF, tumor necrosis factor; CHO, Chinese hamster ovary. P < 0.05 for TNF-α vs. CHO animals for collagen and relative rate of collagen production; n = 3 animals for each group.

indicate that TNF-α inhibits collagen type I production independent of factors related to weight loss (12, 13, 40). The purity of the extracted liver collagen in TNF-α and CHO mice was demonstrated by comparison with collagen standards on PAGE (Fig. 1A, lanes 1 and 2). In addition, the purity of the extracted liver collagen was confirmed by its susceptibility to digestion with highly specific collagenase (13, 50) (Fig. 1A, lane 3). The yield of collagen, although variable, was comparable in CHO and TNF-α mice. In similar purification procedures, we have demonstrated that hepatic collagen had no contaminant proteins as determined by the ratio of hydroxyproline to proline and hydroxyproline (10).

To determine whether the decreased collagen synthesis in the liver of precachectic TNF-α mice was the result of an inhibition of collagen-α1(I) gene expression, we analyzed collagen mRNA levels. Total RNA was purified from the liver of TNF-α and CHO mice, and the abundance of collagen-α1(I) mRNA was measured after solution hybridization with a specific probe by a sensitive RNase protection assay (6–8, 18, 26). As expected, the protected segment of the collagen-α1(I) mRNA was 110 bases, confirming the specificity of the assay. The levels of collagen-α1(I) mRNA (corrected by the steady-state levels of β2-microglobulin mRNA) were decreased in the liver of TNF-α mice (Fig. 1, B and C), indicating a selective effect of chronically elevated TNF-α on collagen-α1(I) gene expression in the liver, similar to that found in cultured fibroblasts and stellate cells (2, 50).

To characterize the TNF-α-responsive region within the collagen-α1(I) gene, we performed experiments with transgenic mice expressing the hGH reporter under the direction of regulatory regions of the human collagen-α1(I) gene (49). We have reported kinetic studies, indicating that in 20- to 25-g mice the intraperitoneal injection of 500 ng of TNF-α results in a peak TNF-α serum level of ~20 ng/ml at 45 min, decreasing to ~4 ng/ml at 3 h (1). These TNF-α values are comparable to those found in cachectic TNF-α mice (5, 7, 8) and in cachectic patients (22, 48, 54). Both the hGH transgene mRNA and the endogenous collagen-α1(I) mRNA were detected by a sensitive and specific RNase protection assay, and measurement of 18S RNA was used as an internal standard (34, 49). In addition, the confounding variable of a different transgene copy number when the two lines were compared (49) was avoided by assessing expression under baseline conditions and after TNF-α treatment in the same transgenic line (see Figs. 2 and 3). TNF-α inhibits collagen-α1(I) gene transcription in cultured human and murine fibroblasts during a 4- to 8-h incubation (50). Similarly, the expression of the −2300 COL-hGH transgene or the −440 COL-hGH transgene (Fig. 2A) was decreased in the liver in parallel to the endogenous collagen gene, 8 h after treatment with human recombinant TNF-α (500 ng at 0 and 5 h) (Fig. 2, B and C). The apparent decreased baseline expression of the hGH in the liver of the −440 COL-hGH transgene compared with the −2300 COL-hGH transgene (−8-fold), reflects a five- to sixfold difference in copy number, as well as a small difference in riboprobe specific activity (26, 34) (Fig. 2C).
Because TNF-α could affect liver collagen-α1(I) gene expression directly and/or indirectly through induction of cytokine cascades in other cells, we analyzed the effects of TNF-α on cis-regulatory regions of the collagen-α1(I) gene in cultured cells. Stellate cells were freshly isolated from −440 COL-hGH transgenic mice, and to prevent their activation and proliferation (27, 32) after enzymatic isolation, cells were cultured with or without treatment for 6 days on an EHS matrix. Under these conditions, cell quiescence was documented by the number of cells in S phase (32) and by [3H]thymidine incorporation (27). In addition, stellate cells cultured on an EHS matrix did not express α-SMA whether or not they were treated with TNF-α (Fig. 3A, left and middle). In contrast, stellate cells were activated when treated with FeSO₄ (50 µM)/ascorbate (200 µM) for 6 days (Fig. 3A, right), which induces oxidative stress as described previously (32). Because primary quiescent stellate cells have a low collagen gene expression (26), we treated these cells with TNF-α for 6 days to facilitate an accurate determination of the cytokine effects. Treatment of stellate cells for 6 days with TNF-α (10 ng/ml) inhibited expression of the −440 COL-hGH transgene to an extent comparable to that induced by TNF-α in animals bearing this collagen-hGH transgene.

Fig. 2. TNF-α inhibits liver expression of human growth hormone (hGH) in transgenic animals bearing −2300 or −440 COL-hGH minigenes. A: schematic representation of human collagen-α1(I)-hGH minigenes. These constructs were used for the generation of transgenic mice. The 5′ flanking regions are shown as open rectangles. Sequences of the first exon and first intron of collagen-α1(I) genes are indicated in solid and hatched rectangles, respectively. Arrows denote start of transcription. Reporter gene sequences of hGH (exons 4 and 5) are indicated in gray rectangles. B: expression of collagen-α1(I)-hGH minigenes. Transgenic mice received intraperitoneal injections of saline (control) or TNF-α as described in METHODS, and collagen-α1(I) mRNA was analyzed by RNase protection assay. At least 3 animals were used for each group in all experiments. Values were corrected for 18S RNA; P < 0.05 for all TNF-α treatments. TNF-α decreased endogenous collagen-α1(I) mRNA in the liver; P < 0.05. C: representative examples of RNase protection assay showing expression of hGH transgenes in control (lanes 1, 2, 5, and 6) and TNF-α (lanes 3, 4, 7, and 8)−treated animals bearing −2300 COL-hGH (lanes 1–4) or −440 COL-hGH minigenes (lanes 5–8). Protected hGH mRNA segment is 168 nucleotides.

Fig. 3. TNF-α inhibits expression of human collagen-α1(I)-hGH minigenes in hepatic stellate cells. A: α-smooth muscle actin (α-SMA) expression in hepatic stellate cells. Hepatic stellate cells were freshly isolated from transgenic animals and cultured on EHS matrix. Representative examples of untreated control (left) or stellate cells cultured on EHS matrix, whereas FeSO₄/ascorbate stimulated α-SMA expression. B: collagen-hGH transgene expression in primary cell cultures. Hepatic stellate cells were freshly isolated from transgenic animals and cultured on EHS matrix as described previously (32). Hoechst-33342 was used as nuclear counterstain. α-SMA expression was negative in control or TNF-α−treated cells cultured on EHS matrix, whereas FeSO₄/ascorbate stimulated α-SMA expression. C: representative examples of RNase protection assay for hepatic stellate cells isolated from −440 COL-hGH transgenic animals showing protected 168 nucleotide hGH band in control (lanes 1 and 2) and TNF-α-treated (lanes 3 and 4) cells.
(Fig. 3, B and C). Values for hGH mRNA were normalized by the internal standard 18S RNA as described previously (26).

The promoter and first intron of the collagen-α3(I) gene are highly conserved among different species, including humans, mice, and rats (15, 35, 43). Therefore, additional characterization of the cis-regulatory region within the collagen 5′ flanking sequences was obtained by transfecting LUC chimeric reporter genes driven by segments of the mouse collagen-α3(I) gene into day 10 stellate cells growing on a collagen type I matrix. Using a transfection-enhancing reagent and lipofectamine, we achieved a high-efficiency transfection for activated stellate cells growing on a collagen type I matrix as described previously (27). To obtain optimal reporter expression, cells were harvested 48 h after transfection. Expression of the LUC reporter containing 2,300 bp or 220 bp of the 5′ flanking region of the mouse α3(I) collagen gene (without the first intron) was inhibited by TNF-α (Fig. 4). The transfection efficiency, as determined with a pLUC vector, was essentially identical in control and TNF-α-treated cells (13.6 ± 1.5 vs. 14.1 ± 2.5 U/mg DNA; not significant), indicating that TNF-α did not decrease collagen-chimeric reporter gene expression spuriously by inhibiting the transfection of these genes.

**DISCUSSION**

We (50) and others (2) have suggested that TNF-α could play an important role in tissue fibrogenesis by inhibiting collagen-α3(I) gene transcription. This study supports the hypothesis that TNF-α inhibits liver collagen-α3(I) gene expression independent of the confounding variables of tissue necrosis and inflammation. In addition, we demonstrated that TNF-α inhibits collagen-α3(I) gene expression in cultured stellate cells, independent of the confounding variables of stellate cell activation or proliferation (16, 20, 21). Although an increase in NF1 (26) and Sp1 (44) binding activities has been reported in stellate cells after their activation, these changes appear to be predominantly quantitative (26, 44).

In this report we show that chronically elevated serum TNF-α inhibits liver collagen-α3(I) gene expression, before the onset of weight loss in these animals (5, 7, 8). To eliminate the confounding variable of weight loss, which is known to downregulate collagen-α3(I) gene expression in scurvy and fasted animals (12, 13, 40, 51), we studied TNF-α-secreting precachectic animals (5, 7, 8). The body weight of precachectic TNF-α animals was essentially equal to that of CHO mice. Here we show that TNF-α decreases the steady-state level of collagen-α3(I) mRNA and collagen synthesis in the liver of precachectic mice, in agreement with the transcriptional inhibition induced by TNF-α in cultured human and murine fibroblasts (50). Similar effects on liver collagen-α3(I) gene expression were observed after only an 8-h treatment with TNF-α, in agreement with the rapid effects of this cytokine on cultured cells (2, 50).

To characterize the regulatory region of the collagen-α3(I) gene responsive to the inhibitory effects of TNF-α, we analyzed the expression of reporter chimeric genes in transgenic mice. Although during CCl4-induced fibrogenesis in the liver expression of the collagen-α3(I) gene requires only the presence of an upstream −440 bp region (26), inhibitory cis elements could be localized in any region of the gene, including 5′, intronic, and 3′ segments (11, 19). We found that TNF-α inhibits expression of the −440 COL-hGH as well as of the −2300 COL-hGH transgene in the liver, indicating that the inhibitory effect of this cytokine is exerted on the −440 bp of the 5′ flanking region and/or the first intron of the collagen-α3(I) gene. TNF-α also inhibited collagen synthesis and gene expression in the skin of precachectic animals; however, this effect required a region of the collagen-α3(I) gene beyond −440 bp (8), suggesting a different inhibition of collagen-α3(I) gene expression by TNF-α in various target tissues. Interestingly, the stimulation of collagen-α3(I) transcription is also regulated in a tissue-specific manner. The −440 bp region is sufficient in the liver, but the region between −440 bp and −2,300 bp is required for the efficient transcription of this gene in skin and tendon (26). To our knowledge, this is the first evidence of a tissue-specific inhibitory mechanism in gene transcription.

Inagaki et al. (29) have identified a TNF-α-responsive element within the −378 to −235 sequence (TbRE) of the collagen α3(I) gene in dermal fibroblasts. The suppressive effect of TNF-α on collagen-α3(I) expression was not mediated by either AP-1 or nuclear factor-κB. Interestingly, transforming growth factor-β (TGF-β) stimulates collagen-α3(I) gene transcription through increasing the binding of a Sp1-containing complex to the same cis-acting TbRE region (28). It has been suggested that an NF1 binding site mediates the tran-
scriptional activation of collagen-α2(I) gene by TGF-β (47). It remains to be determined whether similar convergence of the TGF-β and TNF-α pathways occurs in the collagen-α2(I) gene in hepatic stellate cells. However, in fibroblasts the TGF-β-responsive cis element of the collagen-α2(I) gene (α2(TAE)) is present within the −1627 to −1643 bp region of the gene (45) that is required for responsiveness to TNF-α in the skin (8). The activation of collagen-α2(I) gene transcription of TGF-β through the α2-TAE site is independent of NF1 or AP-2 proteins (46).

Although TNF-α inhibits collagen gene expression (2, 50) and stimulates collagenase activity (17) and collagenase gene expression (6) in cultured cells, TNF-α plays a key role in the development of lung fibrosis induced by silica or bleomycin (41, 42). Moreover, expression of a TNF-α transgene in murine lung causes progressive pulmonary fibrosis (38). The apparent discrepancy of these results could be explained in different ways. First, the models of lung fibrosis are characterized by the development of necrosis and inflammation (38, 41, 42). In our study, the effects of TNF-α on stellate cell collagen gene expression were analyzed independent of necroinflammatory reactions. In support of our results, neither the proliferation nor the activation of primary rat stellate cells, features associated with their collagen production (26, 36, 38, 41), were affected by TNF-α (24). The number of cells remained essentially unchanged after 48 h in control and TNF-α-treated cultures (data not shown).

Another mechanism by which TNF-α could contribute to tissue fibrosis is the inhibition of collagen phagocytosis mediated by decreased collagen binding to the α2β1-integrin receptor (14, 37). Perhaps the nature of a given pathological condition may determine the overall effect of TNF-α on tissue fibrosis. In the presence of intense necroinflammatory reactions, TNF-α could stimulate, directly or indirectly, tissue fibrosis.

In this context, induction of an acute phase response stimulates collagen gene expression and this effect was blocked by interleukin-6 (IL-6) antibodies (23). Although TNF-α could stimulate IL-6 synthesis, TNF-α has many other independent effects (4). Furthermore, we have shown that the inhibition of collagen gene expression in cultured human fibroblasts by TNF-α is not mediated by IL-6 (50). From our study, it can also be concluded that p55 TNF-α1 receptor (TNFR1) activation is sufficient to mediate the effects of TNF-α on collagen-α2(I) gene expression, because human TNF-α, used in our experiments, interacts with the murine TNFR1 but not, apparently, with the murine TNFR2 receptor (33).

Because in the intact animal TNF-α could modulate liver collagen-α2(I) gene expression indirectly through cytokine cascades (4, 53), we tested whether the same cis-regulatory region of the collagen-α2(I) gene contains the TNF-α-responsive element(s) in primary stellate cell cultures. In primary cultures of quiescent stellate cells (to avoid the confounding variables of cell activation and cell proliferation) bearing the −440 COL-hGH transgene, TNF-α inhibited the expression of the hGH reporter to a degree similar to that observed in the −440 COL-hGH transgenic mice. These studies indicate that the −440 bp segment of the 5′ flanking region and/or the first intron contains the TNF-α-responsive element(s). Further characterization of the sequences responsive to TNF-α was obtained by transfection into stellate cells of a chimeric LUC reporter gene. A regulatory region of the collagen-α2(I) gene containing the −220/+110 bp segment in the absence of the first intron was sufficient for the inhibition of LUC reporter expression by TNF-α in transfected primary stellate cells. Whether TNF-α inhibits hepatic collagen gene expression and interferes with liver repair (30) in patients with chronic diseases of the liver and other tissues associated with higher levels of serum TNF-α (4, 22, 36, 48, 52, 54) cannot be inferred from our study.

Taken together, these studies suggest that TNF-α inhibits collagen-α2(I) gene expression by affecting directly or indirectly the interaction of transcriptional factors within the −440 bp of the collagen-α2(I) promoter and/or first intron in hepatic stellate cells in vivo and in culture. In conclusion, our studies suggest that TNF-α affects liver collagen-α2(I) gene expression through a tissue-specific region of this gene different from that required to inhibit collagen gene expression in the skin (8).

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