Transcriptional and Post-transcriptional Regulation of 72-kDa Gelatinase/Type IV Collagenase by Transforming Growth Factor-β1 in Human Fibroblasts

COMPARISONS WITH COLLAGENASE AND TISSUE INHIBITOR OF MATRIX METALLOPROTEINASE GENE EXPRESSION*

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The temporal aspects and mechanisms of the regulation of the matrix metalloproteinase (MMP) 72-kDa gelatinase/type IV collagenase (MMP-2) by transforming growth factor-β1 (TGF-β1) were investigated in early passage human gingival fibroblasts and compared with the regulation of the genes for collagenase (MMP-1) and TIMP, the tissue inhibitor of MMPs. Northern hybridization analyses revealed that 1.0 ng/ml TGF-β1 increased the abundance of MMP-2 mRNA/cell ~1.5-fold at 24 h, an increase similar to that observed in the level of $f^{[35]}$methionine pulse-labeled MMP-2 at 24 h (1.9-fold). At 48 and 72 h, the increase in MMP-2 mRNA abundance remained elevated by 1.5-2.2-fold on a per cell basis whereas TIMP mRNA levels were elevated by up to 3.3-fold. In contrast, the relative levels of collagenase mRNA were reduced by 66-75%. The changes in the MMP-2, collagenase, and TIMP mRNA concentrations in response to TGF-β1 were blocked by cycloheximide indicating that protein synthesis was required to mediate the effects of TGF-β1 on these mRNA levels. TGF-β1 was also found to increase the half-life of the MMP-2 mRNA from ~46 to ~150 h but did not alter the stability of TIMP mRNA ($t_{1/2}$ ~60 h). Nuclear run-off transcription assays revealed that MMP-2 gene transcription was increased ~5-fold 7 h following TGF-β1-treatment but returned to control levels by 24 h. In comparison, increased TIMP gene transcription was only detectable after 24 h whereas collagenase gene transcription, although low in control cells, was undetectable at 24 h. Gene transcription, mRNA levels, and message stability of the genes for the extracellular matrix proteins type I collagen and fibronectin were also increased by TGF-β1. Thus, the similarity in the control of MMP-2, α1(I) procollagen, and fibronectin expression at the transcriptional and post-transcriptional levels indicates that these genes may share regulatory elements. In comparison, TGF-β1 reduced the level of collagenase mRNA and increased the level of TIMP mRNA as a result of altered transcriptional activities, through pathways that required protein synthesis, and without changes in mRNA stability.

The TGF-β1 gene family consists of at least five homologous genes whose products exert a wide range of effects on the differentiation and activity of many cells (reviewed in Refs. 1, 2). Although TGF-β1 can induce a tumorigenic phenotype in some mesenchymal cells when added in combination with epidermal growth factor (3), TGF-β1 appears to be synthesized by all normal cells studied to date (4) and has been localized by immunochemistry (5) and in situ hybridization (6) to many tissues during embryogenesis. TGF-β1 is also particularly abundant in platelets (7) and bone (8). Of special relevance to understanding the control of connective tissue formation and degradation during morphogenesis and other processes such as normal tissue turnover, angiogenesis, and wound healing, TGF-β1 selectively stimulates the synthesis of connective tissue matrix components both in vivo (9, 10) and in vitro (11-16). These effects may be augmented by reducing the synthesis of proteinases that are involved in connective tissue degradation, such as collagenase (17), stromelysin (MMP-3) (18, 19), cathepsin L (20), plasminogen activators (21), and elastase (22). TGF-β1 further reduces proteolytic activity by increasing the expression of plasminogen activator inhibitor-1 (15, 17, 23-25) and TIMP (17, 25), a specific inhibitor of MMPs. TGF-β1 can also modify the effects of other growth factors. For example, TGF-β1 reduces the level of collagenase expression induced by epidermal growth factor and basic fibroblast growth factor and amplifies the induction of TIMP by epidermal growth factor and fibroblast growth factor (26). Together, these actions strongly implicate TGF-β1 as an important modulator of connective tissue homeostasis.

Although the pleiotropic effects of TGF-β1 appear, in general, to promote connective tissue formation, we have demonstrated that TGF-β1 increases the level of MMP-2 in the conditioned medium of human fibroblasts (17) and of primary keratinocytes (18).
cultures of rat bone cells (25), a response that appears to oppose the net formative effects of TGF-β1. This observation is of particular interest since the 72-kDa gelatinase (MMP-2) has been identified as a type IV collagenase (27), a MMP that can cleave native type IV collagen and whose activity is thought to be pivotal in tumor cell invasion and metastasis (28). Moreover, type IV collagenolytic activity is also likely to be an important feature of basement membrane remodeling that occurs during angiogenesis and tissue morphogenesis, processes that are stimulated by TGF-β1 (1, 2).

Three cell surface receptors for TGF-β have been identified, but the mechanism of signal transduction to effect the expression of target genes remains elusive. It does not appear to involve protein phosphorylation, CAMP, phosphoinositol turnover, or Ca²⁺ influx (see Ref. 2). Nonetheless, TGF-β1 exerts both transcriptional and post-transcriptional control of the fibronectin and α1(I) procollagen genes in fibroblasts and osteoblastic cells. In addition to increasing transcription of these genes (15, 29, 30), TGF-β1 acts post-transcriptionally in fibroblasts to stabilize the mRNAs of the α1(I) procollagen and fibronectin genes (15, 29–32). In contrast, TGF-β1 suppresses the transcriptional activity of several genes including stromelysin (18). Recently, a specific palindromic sequence, termed the TGF-β inhibitory element (TIE), has been identified as the recognition sequence for a transcriptional inhibitory complex involving the product of the c-fos proto-oncogene, FOS, in the stromelysin and collagenase promoters (33).

Since TGF-β1 promotes MMP-2 expression, but reduces the levels of the related MMPs collagenase and stromelysin, we have investigated and compared the temporal and mechanistic aspects of TGF-β1 regulation of MMPs in normal human fibroblasts. Our studies demonstrate that TGF-β1 regulates MMP-2 expression at the transcriptional and post-transcriptional levels by inducing an early increase in MMP-2 transcription and an increase in the half-life of the MMP-2 mRNA. In these respects MMP-2 is regulated in a manner more similar to α1(I) procollagen and fibronectin than to other MMPs.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Adult human gingival fibroblasts (8–12th passage) (Gin-1, CRI1292, American Type Culture Collection, Rockville, MD) were cultured and passaged as described previously (17). Cells were grown in a humidified 5% CO₂, 95% air incubator in a-minimal essential medium supplemented with 15% (v/v) fetal bovine serum and antibiotics to a cell density of 6–10 × 10⁵ cells/cm². For experiments, confluent cultures were made quiescent by 24-h serum deprivation (0.2% (v/v) fetal bovine serum, α-minimal essential medium) and then incubated in the continuous presence of 40 pm TGF-β1 (1.0 ng/ml) (R & D Systems, Minneapolis, MN) or vehicle (1.0 mg/ml bovine serum albumin (Pentex bovine albumin, Miles Laboratories, Elkhardt, IN), 4 mM HCl) for up to 72 h. Conditioned medium was harvested and replaced every 24 h with fresh serum-depleted medium containing the appropriate reagents or vehicle.

**Isotopic Labeling, Protein, and MMP-2 Analyses**—Isotopic labeling of secreted protein was achieved by either continuously labeling cells with 10 µCi/ml of [*⁵⁷ᵐ⁺⁺]methionine (1100 Ci/mmol, ICN Radiochemicals, Irving, CA), or by pulsing cultures with 50 µCi/ml of [*³⁵*S]methionine for 30 min, the labeled proteins then being collected after a 4-h chase. Aliquots (10 µl) of the labeled media were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% polyacrylamide minishub gels as described below. Total secreted protein was quantitated at 24, 48, and 72 h by liquid scintillation counting of the [*⁵⁷ᵐ⁺⁺]methionine-labeled medium proteins after exhaustive dialysis (34) of equal volumes of the medium or from the 10% (v/v) trichloroacetic acid, 1% (w/v) tannic acid-insoluble material as described before (17, 35). MMP-2 was identified in the secreted protein fraction by enzymography (see below) and the levels in the conditioned culture medium quantitated after affinity purification of the enzyme by miniaffinity columns of heparin-agaratin-Sepharose as described before (17, 35). Material bound to the gelatin-Sepharose column was eluted first with 1.0 mM NaCl in 50 mM Tris-HCl, pH 7.2 buffer containing 5 mM CaCl₂, 0.5 µg/ml Brij 35 and, after thorough washing of the column with the same buffer, the more avidly bound MMP-2 and fibronectin were recovered after elution with 2.0 mM DTT. SDS-PAGE of the electrophoresis sample buffer (8.0 µg/ml, 80 mg/ml SDS, in Tris buffer, pH 6.8).

**SDS-Polyacrylamide Gel Electrophoresis**—SDS-PAGE was performed in the presence of 0.1 mg/ml SDS (36) using separating gels containing 10% acrylamide and stacking gels containing 4% acrylamide. Samples were electrophoresed either nonreduced or following reduction with 65 mM DTT and heating at 56°C for 20 min. MMP-2 protein bands on fluorographs were quantitated using laser densitometry at 633 nm which followed exposure of dried 2.5-diphenyloxazole impregnated gels at ~70°C to Kodak SB-5 x-ray film for various time selections to be in the linear range of the densitometric response. Enzymograms were performed as described previously (37) using substrate gels containing gelatin at a concentration of 40 µg/ml. After SDS-PAGE, the substrate gels were incubated in 50 mM Tris-HCl, 200 mM NaCl, 5 mM CaCl₂, 0.5 µg/ml Brij 35, pH 7.2, at 37°C for 2 h. The gels were fixed and then stained in 0.1% (w/v) Coomassie Blue G-250, 200 mM H₂PO₄, 50 mg/ml ammonium sulfate, pH 2.5, with 20% (v/v) methanol and 5% (v/v) acetic acid. The dried gels were visualized, then washed with 20% (v/v) methanol and 20% (v/v) acetic acid, and clarified by soaking in the aqua blue-stained substrate gel. The following proteins were electrophoresed under reduced conditions as relative molecular mass marker proteins: myosin (200 kDa), β-galactosidase (116.5 kDa), phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), myoglobin tryptic inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa).

**RNA Extraction and mRNA Half-life Determination**—Cellular RNA from confluent cultures grown in 100-mm tissue culture dishes was extracted in LiCl as described before (17). Following phenol–phenol-chloroform purification, the RNA yields were determined for each sample by spectrophotometric analysis of 1/10th of the final sample volume. To estimate the half-lives of specific mRNAs, cells were treated as described above with either TGF-β1 or vehicle and after 24 h of incubation 60 µM 5,6-dichloro-1-β-D-ribofuranosyl benzimidazole (DRB, Sigma), a specific RNA polymerase II inhibitor, was added to the cultures. Then the RNA was then extracted at each time point (1.5, 3, 6, 16, and 24 h) and analyzed after slot-blotting and hybridization with appropriate cDNA probes (see below). To determine the involvement of newly synthesized protein in the modulation of specific mRNA levels in response to TGF-β1, 100 µg/ml cycloheximide was added to cell cultures 15 min before the addition of 1.0 ng/ml TGF-β1. RNA was extracted at several time points (3, 5, 7, 12, and 24 h) following the addition of TGF-β1 or vehicle and analyzed by slot-blotting and hybridization with appropriate cDNA probes (see below). As judged by cell morphology and cell numbers, the dose of cycloheximide was not toxic over the time intervals studied.

**Northern Hybridization and Slot-blot Analyses**—Aliquots of extracted cell RNA (5 µg) were fractionated on 1.2% (w/v) agarose, 2.2 M formaldehyde gels and transferred onto BioTran membrane (0.22-μm pore size) (ICN, Costa Mesa, CA) using a Vacujob apparatus (Pharmacia LKB Biotechnology Inc.). In other experiments, after ensuring the specificity of the cDNA probes by Northern blot analysis, 5 µg of RNA was spotted onto BioTran membrane using a Scheicher & Schuell slot-blot apparatus according to the manufacturer’s instructions. Following UV cross-linking, the blots were hybrized in 50% (v/v) formamide, 5 × SSC (standard saline citrate), 5 × Denhardt’s solution (1 × Denhardt’s = 200 µg Ficol, 200 µg/ml polyvinylpyrrolidone, and 200 µg/ml bovine serum albumin), 25 mM sodium phosphate, pH 6.5, 2.0 mg/ml SDS, and 200 µg/ml denatured salmon sperm DNA (Sigma). Hybridization was performed for 18 h with [*³²P*]dCTP-labeled cDNA probes at a concentration of ~5.0 × 10⁶ cpm/300 cm² membrane in 15 ml of prehybridization solution. After hybridization, the blots were washed in 2 × SSC, 1 mg/ml SDS at 22°C and then for 30 min with two changes of 0.2 × SSC, 1 mg/ml SDS at 65 or 60°C. Blots were autoradiographed at ~70°C using Kodak X-Omat AR X-ray film with two intensifying screens and quantitated by laser densitometry. The loading of equal amounts of RNA was confirmed by staining the gels with ethidium bromide and visualization of the ribosomal bands under UV light. The data was normalized for the amount of RNA loaded as determined from pho- tolmetric analysis of the ethidium-stained bands (see below) and scanned densitometrically and the data adjusted for the RNA yield from each corresponding RNA preparation to give the total yield of
specific mRNA/cell. Experiments were performed in triplicate and the data expressed as the mean ± S.D.

Preparation of cDNA Probes—CsCl banding was used to purify supercoiled plasmids containing the cDNA inserts listed below. Plasmids were cleaved by restriction digestion using the appropriate restriction enzymes and the cDNA insert separated from the vector after fractionation on 1.2% (w/v) agarose gels. Geneclean (Bio 101, CA) was used to concentrate the excised cDNAs before labeling by random priming with [32P]dCTP (>3000 Ci/mmol, Amersham Corp.) to a specific activity of ~5 × 10^6 cpm/μg cDNA prior to hybridization.

The cDNAs used were kindly provided as follows. A 1.7-kb EcoRI fragment of the human collagenase mRNA cDNA was from Dr. C. Brinkerhoff, Dartmouth Medical School Hanover, N.H.; human MMP-2 cDNA was from Dr. G. I. Goldberg, Washington University School of Medicine, St. Louis, MO; a 0.7-kb BamHI, Accl fragment of murine TIMP cDNA was from Dr. D. Denhardt, Rutgers University, Piscataway, N.J.; human fibronectin cDNA was from Dr. F. Ramirez, Rutgers University, Piscataway, N.J.; murine SPARC (secreted protein, acidic, and rich in cysteine) cDNA was from Dr. B. Hogan, Vanderbilt University, Nashville, TN; human TGF-β1 cDNA was from Dr. R. Derynck, Genentech; both a 1.8-kb EcoRI fragment of human stromelysin cDNA and a 1.2-kb EcoRI fragment of human putative metalloproteinase-1 was from Dr. R. Brethnach, Institut de Chimie Biologique, Strasbourg Cedex, France; and a 1.7-kb fragment of murine interleukin 1 cDNA was from Dr. U. Guhler, Hoffmann La Roche, Nutley, NJ. Also used were the cDNA for porcine osteopontin (38) and a Fnu4HI-DraI cDNA fragment derived from the 3’ end of the porcine α1(Ⅰ) procollagen cDNA that is 90% identical to the human α1(Ⅰ) procollagen (15, 54). The α1(Ⅰ) procollagen cDNA probe hybridizes to both of the two α1(Ⅰ) procollagen mRNAs that are present in RNA extracted from most cells.

Transcription Analysis—Nuclear run-off transcription assays were performed as described before (35). In brief, triplicate 100-mm dishes of confluent quiescent cultures were treated with 1.0 ng/ml TGF-β1 or vehicle in serum-deficient medium for 7 or 24 h. Nuclei were isolated by lysis buffer consisting of 5 μl/ml Nonidet P-40, 150 mM KCl, 5 mM MgCl2, 250 mM sucrose (DNase-, RNase-free), 50 mM Tris-HCl, pH 7.4, containing 1 mM phenylmethylsulfonfyl fluoride (freshly prepared in 95% (v/v) ethanol), then washed with the lysis buffer, and twice with transcription buffer (50 mM NaCl, 4 mM MnCl2, 0.25 mM EDTA, 350 mM (NH4)2SO4, 120 mM Tris-HCl, pH 7.8, 34 units/ml RNAguard (Pharmacia)). Nuclei were resuspended in transcription buffer, a further 10 units of RNAguard added, and the nascent RNA transcripts run-off and labeled for 45 min at 32 °C by addition of 15 μl of ribonucleotide solution (1 mM ATP, 1 mM CTP, 1 mM GTP, 3.5 μM UTP plus 125 μM [32P]UTP (~3000 Ci/mmol, Amersham Corp.)). RNA was extracted as described above, denatured by heating at 65°C in 50% (v/v) formamide for 10 min, and the labeled RNA was hybridized for 96 h at 42 °C to cDNA immobilized on BioTrans membrane. Quantities of DNA used were as follows: 1.0 μg for CsCl-purified cDNA in plasmid form and 0.1 μg for cDNA excised and purified from the vector. The membranes were washed (0.1 × SSC, 1.0 μg/ml SDS at 55 °C), dried, and exposed to Kodak X-OMAT AR X-ray film at −70 °C for 10–14 days using two intensifying screens.

RESULTS

Although TGF-β1 characteristically increases the levels of total cell RNA (32), the synthesis of total secreted protein, and the connective tissue matrix components type I collagen, fibronectin, and SPARC in fibroblasts derived from several species (10–16), TGF-β1 did not change the expression of α1(Ⅰ) procollagen, α2(Ⅰ) procollagen, and fibronectin mRNAs in late passage (24–34th passage) fibroblasts (26). To ensure that the human gingival fibroblasts (8–10th passage) used in our experiments responded to TGF-β1, total secreted protein, and the yields of total RNA and the mRNAs for α1(Ⅰ) procollagen, fibronectin, and SPARC in response to 1.0 ng/ml TGF-β1 were determined after 24, 48, and 72 h in culture. TGF-β1 increased total secreted protein from 1.4- to 1.8-fold and total cell RNA from 1.5- to 2.8-fold (Fig. 1) while cell numbers, determined by Coulter counting, were essentially unaltered (~3.5 × 10^6 cells/100-mm culture dish). In addition, following the daily administration of 1.0 ng/ml TGF-β1, matrix protein

![Fig. 1. Quantitation of cellular RNA yields and secreted protein synthesis in response to TGF-β1 treatment.](image-url)

![Fig. 2. Northern hybridization analysis of extracellular matrix gene expression.](image-url)
expression was differentially stimulated as observed previously (15). The stimulation of SPARC (4.0-fold at 9 h), type I collagen (2.6-fold), and fibronectin (2.4-fold) synthesis at 24 h (data not shown) reflected an increase in the levels of the respective mRNAs for these proteins at 24 h (Fig. 2). Further, in the presence of TGF-β1 the mRNA levels of these genes remained elevated at 72 h compared with control cell mRNA levels. Of note, unlike many human cells, human gingival fibroblasts produce the 4.7-kb α1(I) procollagen mRNA almost exclusively and essentially none of the minor polyadenylated mRNA species of α1(I) procollagen (15). However, both polyadenylated forms of SPARC mRNA were produced. These experiments confirmed that the response of the human gingival fibroblasts to TGF-β1 was typical of normal mesenchymal cells.

To investigate the effects of TGF-β1 on MMP-2 expression, the proteinase was affinity purified on gelatin-Sepharose from equal aliquots of conditioned medium. The purified MMP-2 was identified by enzymography and by a characteristic electrophoretic mobility shift from 72 kDa, when electrophoresed reduced, to 66 kDa, when electrophoresed nonreduced (Fig. 3). The amount of MMP-2 secreted into the conditioned culture medium, determined by quantitation of the affinity purified protein, was increased 1.8-fold at 48 and 72 h (Fig. 3) and data not shown), but as found before (17), the amount of MMP-2 that accumulated in the conditioned medium during the first 24 h of TGF-β1 treatment was increased only slightly. That TGF-β1 was stimulating the synthesis of MMP-2 that accumulated in the conditioned medium during the first 24 h of TGF-β1 treatment was shown by pulse-chase experiments (Fig. 4). After 24 h of TGF-β1 treatment, the synthesis and secretion of pulse-labeled MMP-2 into the chase medium was found to occur in a dose-dependent manner with a maximal increase (1.9-fold) being obtained at 1.0 ng/ml TGF-β1. The electrophoretic profiles obtained from these experiments also showed a selective, dose-dependent increase in procollagen, fibronectin, and plasminogen activator inhibitor-1 synthesis, emphasizing further the differential stimulation of secreted proteins by TGF-β1.

Although these experiments showed that MMP-2 synthesis...
was elevated 1.9-fold on a per cell basis by TGF-β1, the level of MMP-2 mRNA compared to the total RNA was not changed appreciably (Fig. 5). However, the total cell RNA was increased 1.5–2.8-fold after TGF-β1 treatment, even though cell numbers remained constant (Fig. 1). Therefore, the data was normalized for the yield of total cell RNA from each corresponding RNA preparation. As shown in Table I, TGF-β1 increased the abundance of MMP-2 mRNA by 1.5–2.2-fold on a per cell basis over 24–72 h, an increase that corresponded closely with the 1.9-fold increase in secreted MMP-2 protein. In contrast to the effects of TGF-β1 on MMP-2 mRNA levels, the relative levels of collagenase mRNA were reduced by 66–75% (Fig. 5). However, when calculated on a per cell basis (Table I) the reduction in collagenase mRNA levels was less than originally measured and ranged from 50–60% compared with controls.

The mechanisms by which TGF-β1 affected the changes in MMP-2 and collagenase mRNA levels were investigated further. When TGF-β1 was administered to cultures 15 min after the addition of the protein synthesis inhibitor, cycloheximide, there was complete abolition of the TGF-β1-induced increase in MMP-2 mRNA levels at 24 h (Table I). Similarly, cycloheximide blocked the reduction in collagenase mRNA levels induced by TGF-β1, indicating that protein synthesis was essential to mediate the effects of TGF-β1 on both MMP-2 and collagenase mRNA levels.

Since TGF-β1 is known to increase the levels of α1(I) procollagen and fibronectin mRNAs partially through increased mRNA stability (15, 31, 32), the effect of TGF-β1 on the stability of the MMP-2 and collagenase mRNAs were investigated. Following the addition of the RNA polymerase II inhibitor, DRB, to block transcription, the decrease in the levels of MMP-2 and collagenase mRNAs were measured following treatment of quiescent cultures with TGF-β1 (Fig. 6). In control cells, the half-life of MMP-2 mRNA was estimated to be ~46 h. After TGF-β1 treatment the stability of the MMP-2 mRNA was increased with an estimated half-life of ~150 h indicating that decreased MMP-2 mRNA degradation contributed to some of the observed TGF-β1-induced increase in MMP-2 mRNA abundance. In contrast, the half-life of collagenase mRNA, estimated to be ~53 h, was not increased.

### Table I

**Quantitation of MMP-2, collagenase, and TIMP mRNA abundance in human fibroblasts incubated in the presence or absence of TGF-β1**

| mRNA         | Treatment                      | 24 h                | 48 h                | 72 h                |
|--------------|--------------------------------|---------------------|---------------------|---------------------|
| MMP-2        | Control                        | 1079 ± 71           | 1061 ± 269          | 910 ± 197           |
|              | TGF-β1                         | 1618 ± 127          | 2380 ± 603          | 1688 ± 371          |
|              | Control + cycloheximide        | 1000 ± 107          | ND                  | ND                  |
|              | TGF-β1 + cycloheximide         | 1081 ± 110          | ND                  | ND                  |
| Collagenase  | Control                        | 3374 ± 1003         | 2232 ± 206          | 3056 ± 1239         |
|              | TGF-β1                         | 2035 ± 167          | 1558 ± 82           | 1251 ± 40           |
|              | Control + cycloheximide        | 837 ± 23            | ND                  | ND                  |
|              | TGF-β1 + cycloheximide         | 756 ± 56            | ND                  | ND                  |
| TIMP         | Control                        | 2251 ± 1062         | 1535 ± 396          | 1866 ± 470          |
|              | TGF-β1                         | 3376 ± 922          | 5110 ± 1710         | 4195 ± 720          |
|              | Control + cycloheximide        | 1139 ± 111          | ND                  | ND                  |
|              | TGF-β1 + cycloheximide         | 1165 ± 576          | ND                  | ND                  |

*ND, not determined.

**Fig. 6. Effects of TGF-β1 on the stability of MMP-2 and TIMP mRNA in human fibroblasts.** Fibroblasts were incubated in the presence or absence of 1.0 ng/ml TGF-β1. After 24 h the RNA polymerase II inhibitor DRB (60 μg/ml) was added and the cultures were incubated for the indicated additional times (h). Total cell RNA was then extracted and 5-μg aliquots were analyzed by slot-blot hybridization with [32P]dCTP-labeled cDNA fragments of the indicated genes as detailed under "Experimental Procedures." Autoradiographs of the slot-blot were quantitated using laser densitometry and the results were presented as a semilogarithmic plot of the mean ± S.D. (n = 3).
affected significantly by the treatment with TGF-β1 (data not shown).

TGF-β1 increases the steady-state levels of TIMP mRNA in human fibroblasts (17) and in rat calvarial bone cells (25) and acts synergistically with epidermal growth factor and fibroblast growth factor to increase TIMP expression (26).

To determine whether the TGF-β1-induced increases in TIMP mRNA levels resulted from decreases in mRNA degradation, mRNA stability determinations of the TIMP transcript were also performed (Fig. 6). However, no change in the half-life of TIMP mRNA from ~60 h was found after TGF-β1 treatment.

In addition, cycloheximide added to cultures immediately prior to TGF-β1 was found to block the increase in TIMP mRNA levels (Table 1). Taken together, these data indicated that TGF-β1 exerted its effects on TIMP expression at the transcriptional level.

That TGF-β1 also regulates the expression of MMP-2, collagenase, and TIMP transcriptionally was shown by nuclear run-off assays. The basal rate of transcription of these genes in unstimulated cells was compared with that of fibroblasts incubated with TGF-β1 for 24 h (Fig. 7). Since it was not known when the transcriptional activity of these genes was altered by TGF-β1, the assays were also repeated at 7 h to detect any early changes. Quantitative analysis of nuclear run-off experiments (n = 3 for each time point) showed that TGF-β1 caused an ~5-fold increase in the rate of MMP-2 transcription at 7 h which then returned to near control levels after 24 h. The rates of transcription of the α1(I) procollagen and fibronectin genes were also increased ~4- and ~10-fold, respectively, after 7 h, and by ~5- and ~3.6-fold, respectively, after 24 h of TGF-β1 treatment. Although TGF-β1 increased TIMP transcription, unlike MMP-2, α1(I) procollagen and fibronectin, this was only apparent 24 h after TGF-β1 treatment. Photographic reproduction of faint bands on these blots for presentation was difficult, but the collagenase bands on control cell blots could be scanned, from which it was determined that collagenase transcription was reduced to undetectable amounts after TGF-β1 treatment. Neither stromelysin gene transcription nor stromelysin mRNA (not shown) were evident in either the control cell cultures or in cells treated with TGF-β1.

We also investigated the autoactivation of TGF-β1 transcription and the change in transcription of interleukin 1 by TGF-β1. TGF-β1 induced an increase in TGF-β1 transcription at 7 h which returned to undetectable basal levels at 24 h. The rate of interleukin 1 transcription, which was elevated ~2-fold at 7 h, was further increased by TGF-β1 at 24 h.

**DISCUSSION**

TGF-β1 acts in a concerted manner to promote connective tissue formation by stimulating the synthesis of extracellular matrix components (9-16) and by suppressing overall proteolytic activity through reduced protease synthesis (17-22, 26) and by increased inhibitor expression (17, 23-26). Therefore, it was intriguing to discover that TGF-β1 increases the levels of MMP-2 secreted by human fibroblasts and rat bone cells (17, 25). In an attempt to determine the basis of this seemingly paradoxical effect of TGF-β1, the mechanism of MMP-2 regulation was investigated. The total cellular abundance of MMP-2 mRNA was found to increase from between 1.5-2.2-fold; an amount similar to the increase seen in the synthesis of MMP-2 protein. By happenstance, the increase in MMP-2 mRNA abundance was also similar to the average increase in total RNA induced in these cells by TGF-β1. However, MMP-2 transcription was transiently increased ~5-fold 7 h after TGF-β1 was added to the cells and then returned to control levels by 24 h. Thus, the increase in MMP-2 expression was not due to a nonspecific increase occurring over 72 h.

The effects of TGF-β1 on MMP-2 expression contrast the actions of TGF-β1 on the mRNA levels and gene transcription of the related MMP collagenase. TGF-β1 produced a decrease both in the amount of collagenase mRNA relative to total RNA and in the total abundance of collagenase mRNA calculated on a per cell basis, supporting the results of our previous studies in which functional collagenase was reduced in human gingival fibroblasts by TGF-β1 (17). The pulse of MMP-2 transcription also differed from the pattern of α1(I) procollagen and fibronectin gene transcription which, unlike MMP-2, remained elevated at 24 h, indicating that TGF-β1 increases the expression of these genes through different mechanisms.

The half-lives of stable mRNAs cannot be readily determined since prolonged treatment (>24 h) of cells with RNA polymerase inhibitors may reduce the expression of housekeeping genes and proteins involved in mRNA stability. This in turn may either directly or indirectly affect the half-lives of the individual species of RNA. Therefore, care must be taken in extrapolating data collected up to 24 h. The ~3-fold increase in the stability of the MMP-2 mRNA induced by TGF-β1 would augment the elevated levels of MMP-2 mRNA produced by increased MMP-2 transcription. Further, the increased stability of the MMP-2 mRNA would maintain an elevated expression of MMP-2 over an extended period following the removal of TGF-β1. Similarly, although collagenase transcription was decreased by TGF-β1, given the long half-life of collagenase mRNA and which was not altered by TGF-β1, the reduction in collagenase transcription is reflected in a gradual decrease in collagenase mRNA levels over a long time period. As shown from experiments in which the protein synthesis inhibitor, cycloheximide, blocked the TGF-β1-induced increase in MMP-2 and TIMP mRNAs and the decrease in collagenase mRNA levels, the effects of TGF-β1 on

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**Fig. 7. Nuclear run-off transcription analysis of TGF-β1 effects on human fibroblast gene transcription.** Quiescent fibroblasts (~3.3 × 10⁶) were incubated with 1.0 ng/ml TGF-β1 or vehicle (Control) for 7 or 24 h. Nuclei were isolated and the nascent RNA chains run-off as described under "Experimental Procedures." Equal aliquots of the [α-32P]UTP-labeled transcripts (7 h, 3 × 10⁶ dpm; 24 h, 8 × 10⁶ dpm) were hybridized at 42°C for 96 h with membranes on which the indicated cDNAs or plasmids were immobilized. Osteopontin (opn), which is not normally synthesized by untransformed fibroblasts, and the plasmid pTT73 (Pharmacia) were included as controls. α1(II), α1(I) procollagen; fn, fibronectin; il-1, interleukin 1. Representative autoradiographs that were exposed for 10 days from experiments performed in triplicate are presented.
these mRNAs required de novo protein synthesis. Since TGF-β1 acted both transcriptionally and post-transcriptionally to elevate MMP-2 expression, this indicates that the synthesis of labile proteins is required to either increase MMP-2 transcription or to act post-transcriptionally to stabilize MMP-2 mRNA, or both.

Although Brinkerhoff et al. (39) has found that the levels of collagenase mRNA were increased in response to phorbol ester treatment through increased mRNA stability, control of mRNA degradation does not appear to be a general mechanism of MMP regulation. Concanavalin A, which is a potent inducer of collagenase (35), does not increase the stability of collagenase or MMP-2 mRNAs (35). Further, the reduction in collagenase mRNA levels resulting from retinoic acid and dexamethasone treatments also occurs in the absence of changes in mRNA stability (39). We have found here that the TGF-β1-induced reduction in the levels of collagenase mRNA occurred in the absence of mRNA stability changes. Although some regulatory elements that contribute to mRNA stability reside in the 3' untranslated region of mRNA, the mechanism whereby mRNA stability is regulated is not well understood. Notably however, an AUUUA sequence that has been implicated in the stabilization of lymphokine and oncogene mRNAs after TPA induction (40) is present in the 3'-untranslated region of MMP-2 mRNA.

It is significant that, despite the modest increase in MMP-2 expression induced in these cells by TGF-β1, the increase is in marked contrast to the effects of TGF-β1 on the expression of all other proteinases reported to date including collagenase (17, 25, 26), stromelysin (18, 19), the plasminogen activators (21), cathepsin L (20), and elastase (22). This underscores the importance of the biological role of MMP-2 in matrix formation and remodeling in vivo such as that occurring during morphogenesis, angiogenesis, and wound healing, processes that are stimulated by TGF-β1 (1, 2).

We have previously shown that TGF-β1 increases the synthesis of immunoprecipitable and functional TIMP protein and the levels of TIMP mRNA in human fibroblasts (17) and rat bone cells (25). Here, we have shown that TGF-β1 increases TIMP mRNA levels as a result of increased transcription of the TIMP gene, through pathways that required the synthesis of new protein, and without any apparent changes in TIMP mRNA stability. Of note, the 3' end of TIMP mRNA does not contain an AUUUA element. The induction of TIMP transcription by TGF-β1 was delayed compared to that of the α1(I) procollagen, fibronectin, and MMP-2 genes, which exhibited a high level of transcription 7 h after TGF-β1 treatment. Moreover, unlike these genes, TGF-β1 regulated TIMP expression without changes in mRNA stability further showing that TGF-β1 acts through several different mechanisms to modulate gene expression. Since increased TIMP transcription by TGF-β1 was only apparent after 24 h, this could account for the delayed onset in TIMP protein increases in the conditioned medium of TGF-β1-treated human fibroblasts reported previously (17). This may also explain the failure of Edwards et al. (26) to detect any direct effects of TGF-β1 alone on TIMP mRNA expression in 12-h time course experiments and on functional TIMP in 24- and 48-h conditioned medium. Nonetheless, Edwards et al. (26) showed that the induction of TIMP mRNA by epidermal growth factor and fibroblast growth factor was amplified at 12 h by TGF-β1.

In consideration of the present and previous data (17, 25) and that of Edwards et al. (26), it appears that with increasing subculture, cells may lose their responsiveness to TGF-β1 in terms of modulation of connective tissue formation. Using 8-12th passage human fibroblasts (this study and Ref. 17) and primary cultures of rat calvarial cells (25), TGF-β1 has been shown to regulate collagenase and TIMP expression. In contrast, Edwards et al. (26) showed with human fetal lung fibroblasts (MRC-5 cells) in late passage (subculture 24–34), that TGF-β1 alone did not alter the cellular expression of either collagenase or TIMP, nor indeed of type I collagen and fibronectin, matrix proteins that are characteristically increased by TGF-β1 (11–16). Nonetheless, TGF-β1 could still modify MRC-5 cellular activity in response to other growth factors (26). Recently, a specific 10-base pair sequence, TIE, was identified in the 5'-flanking region of the stromelysin and collagenase genes as a novel FOS-binding site that is involved in the TGF-β1-induced repression of transcription (33). Since senescent cells show transcriptional repression of c-fos induction (41), this may contribute to the reduced efficacy of TGF-β1 on cells at high passage number. The loss of the TGF-β1-induced decrease in collagenase expression following cycloheximide treatment is also consistent with the requirement for FOS to mediate the TGF-β1 effects on collagenase transcription. Moreover, in accordance with our findings that TGF-β1 increased MMP-2 transcription, we found no TIE boxes in a search of the first 290 base pairs of the 5'-flanking region of the MMP-2 gene sequence published by Frisch et al. (42).

In response to other agents, the regulation of MMP-2 also appears distinct and differs from the regulation of collagenase and stromelysin, which are coordinately regulated by a variety of stimuli including TPA, interleukin 1, and cell shape changes (43–45). For example, concanavalin A differentially stimulates collagenase and MMP-2 expression by ~30- and ~3-fold, respectively (35). The MMP-2 promoter also lacks a TPA response element (42), consistent with the observations that TPA, a well-characterized inducer of collagenase and stromelysin expression, does not induce MMP-2 expression in normal human fibroblasts (27, 35).

Unlike epidermal growth factor, fibroblast growth factor, interleukin 1, and TPA which stimulate both TIMP and collagenase expression coordinately (26, 35, 43, 46), TGF-β1 regulates TIMP and collagenase expression in a reciprocal manner. Moreover, Stetler-Stevenson et al. (47) have recently shown that unlike TIMP, which is up-regulated by TGF-β1, TIMP-2 is down-regulated by TGF-β1. Since TIMP-2 is complexed to latent MMP-2 (48, 49) and may regulate the activation of MMP-2 from a 62-kDa (−DTT) activation intermediate to the high specific activity 59-kDa form (−DTT) (35), the reduction in TIMP-2 expression by TGF-β1 would be expected to facilitate MMP-2 activation and activity. This, taken together with our recent findings which have shown that concanavalin A increases collagenase but suppresses TIMP and TIMP-2 expression (35), reveals that fibroblasts can be regulated to achieve either a formative or a resorptive phenotype. Further, the opposite regulation of MMP-2 and collagenase by TGF-β1 further demonstrates that distinct regulation of MMPs and indicates that precise, independent control of MMP expression is possible which may be important in their regulation during different physiological and pathological processes. While it is unlikely that the changes in the net levels of MMPs and TIMP induced by TGF-β1 are solely responsible for the major tissue changes that occur during morphogenesis, the reduced proteolytic activity of cells would tend to favor the deposition and maturation of connective tissue matrices.

TGF-β1 increased total secreted protein synthesis nearly 2-fold. However, as found before, the effects of TGF-β1 were selective; not all protein, or RNA species, were elevated by the same amount or at the same time (14–17). Some proteins,
for example type I collagen, fibronectin, and SPARC, are increased more than the amounts of total secreted protein induced by TGF-β; others, for example MMP-2, are stimulated to a similar level as that found for the increase in total protein, whereas the levels of collagenase and stromelysin are reduced. TGF-β1 elevated the mRNA levels of αI(1) procollagen and fibronectin both through an early increase in transcription and, in agreement with two previous reports (31, 32), through increased mRNA stability (ΔG2 fibronectin mRNA increased from 8 to >24 h and ΔG2 αI(1) procollagen mRNA increased from 9 to 20 h following TGF-β1 treatment (15)). Thus, the early induction of MMP-2 gene transcription at 7 h and the increased stability of the MMP-2 mRNA are similar to the regulation of the matrix proteins type I collagen and fibronectin but contrast the TGF-β1 regulation of other MMPs. Therefore, MMP-2 may share with αI(1) procollagen and fibronectin DNA regulatory elements in the promoter/enhancer regions that regulate transcription and show similarities in the 3′-untranslated region of their RNAs where sequences that influence mRNA degradation reside (50).

Since TGF-β1 is considered to be an important regulator of morphogenesis and in the remodeling of growing and mature tissues (2), the coordinated regulation of MMP-2 with type I collagen, fibronectin, and SPARC, a protein found in high amounts in platelets (51), embryonic tissue (52), and rapidly remodeling tissues (53), also argues strongly for an important role for MMP-2 in the establishment of extracellular matrices during embryogenesis, remodeling, and wound healing. For example, the removal of abnormal or unfolded collagen that may retard collagen fibril formation and the remodeling of existing or newly deposited basement membrane type IV collagen may be important functions of MMP-2. Similarly, the selective induction of MMP-2, but not of other MMPs by TGF-β1, may favor type IV collagenolysis which would facilitate basement membrane remodeling in tissue morphogenesis and vascular outgrowth near and through basement membranes during angiogenesis. Of note, the in situ hybridization studies of Lehner and Akhurst (6) have revealed that TGF-β1 mRNA is expressed in epithelia but not in the adjacent mesenchyme of the submandibular gland, hair follicles, heart valves, and in tooth buds. Epithelial synthesis of TGF-β1 may stimulate mesenchymal MMP-2 expression and, together with reduced TIMP-2 expression, could facilitate the basement membrane remodeling and morphogenesis in these structures. Indeed, the presence of TGF-β1 mRNA in developing tooth buds (6) and both active and latent MMP-2 in mineralizing enamel (37) is consistent with this proposal.