Independent Regulation of Collagenase, 72-kDa Progelatinase, and Metalloendoproteinase Inhibitor Expression in Human Fibroblasts by Transforming Growth Factor-β*

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The effects of transforming growth factor-β (TGF-β) on fibroblast collagenolytic activity were investigated to determine if modulation of matrix metalloendoproteinase activity could augment the stimulation of connective tissue formation by TGF-β. Quiescent human fibroblast cultures were incubated in the continuous presence of 1.0 ng/ml (40 pm) TGF-β in culture medium supplemented with 0.2% (v/v) serum and containing [35S]methionine. Aliquots of conditioned cell culture media, harvested daily for 4 days, were processed individually to separate procollagenase and a 72-kDa progelatinase from metalloendoproteinase inhibitor (TIMP) and plasminogen activator inhibitor (PAI-1) using tandem minicolumns of heparin- and gelatin-Sepharose. The fractionated 54-kDa procollagenase was quantitated, after p-amino-phenylmercuric acetate activation, by functional assays using soluble [14C]glycine-labeled collagen as substrate. In cultures treated with TGF-β, procollagenase expression was progressively decreased (~50% on day 1, ~75% on day 2) to undetectable levels on days 3 and 4. This decrease occurred despite a 1.6-fold increase in the synthesis of total secreted protein. Contrasting the effect on procollagenase, TGF-β increased the synthesis of a 72-kDa progelatinase (characterized as a matrix neutral metalloproteinase and likely to be MMP-2) up to 1.8-fold, as determined by quantitation of affinity-purified radiolabeled protein and by enzymography. TIMP biosynthesis was analyzed by immunoprecipitation and quantitated by functional assays for biologically active TIMP following fractionation of the conditioned medium. During the first 24 h TGF-β had little apparent effect on TIMP activity in the medium although the TIMP mRNA transcript was induced 1.3-1.4-fold. Subsequently, TIMP levels were increased 1.7-fold relative to control cells on day 4. This was accompanied by a 2.4-fold increase in TIMP mRNA, indicating that the regulation of TIMP mRNA and protein levels may be a secondary response to TGF-β. In comparison, the synthesis of the M, 48,000 PAI-1, analyzed by [35S]methionine labeling and immunoprecipitation, was elevated >10-fold by TGF-β at all time points with the highest levels occurring at day 2. Thus, the effects of TGF-β on procollagenase, 72-kDa progelatinase, TIMP, and PAI-1 were selective and showed temporal differences. The suppression of procollagenase and the stimulation of TIMP and PAI-1 synthesis are consistent with the promotion of connective tissue matrix formation by TGF-β; whereas the stimulation of 72-kDa progelatinase synthesis may reflect an important function for this neutral metalloendoproteinase in the removal of abnormal or unfolded collagen from wound sites and from newly synthesized extracellular matrix.

The remodeling of connective tissue that occurs during inflammation, bone resorption, fracture repair, and wound healing involves both the degradation of the extracellular matrix and the coordinated synthesis of new matrix components (reviewed in Ref. 1). Several related neutral metalloendoproteinases that can degrade matrix components have been implicated in these processes. Tissue collagenase (EC 3.4.24.7, matrix metalloendoproteinase 1 (nomenclature according to Okada et al. (8))) is synthesized in a latent form (procollagenase) that, on activation, can cleave the native triple helical region of group 1 interstitial collagens into characteristic ⅓- and ⅒-collagen degradation fragments (2) that can be further degraded by other matrix metalloendopeptinases. Matrix metalloendopeptinase-5 (3) can cleave native ⅒-collagen fragments and the denatured α-chains of collagen can be further degraded by gelatinase (matrix metalloendopeptinase-2) (4, 5) and collagenase itself (6). Stromelysin (matrix metalloendopeptinase-3), a proteinase having a wider substrate specificity, also has activity against gelatin and is active on fibronectin, proteoglycan core protein, and laminin (7, 8). Telopeptidase (matrix metalloendopeptinase-4) (9) may also facilitate collagenolysis by the cleavage of the carboxyl-terminal telopeptide region of collagen. Cellular control of these proteolytic enzymes is exerted at several levels including their synthesis and secretion, activation, and inhibition by the stoichiometric complexing of the specific matrix metalloendopeptinase inhibitor TIMP, to the activated enzymes (10, 11). Discerning the regulatory mechanisms and factors that control tissue breakdown and the synthesis of new matrix is central to the understanding of connective tissue homeostasis.

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1The abbreviations used are: TIMP, tissue inhibitor of metalloendopeptinases; APMA, p-amino-phenylmercuric acetate; BSA, bovine serum albumin; DTT, dithiothreitol; EGF, epidermal growth factor; NEM, N-ethylmaleimide; p-gelatinase, 72 kDa-progelatinase; p′ gelatinase, the APMA-activated form of 72 kDa-progelatinase; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; TGF-β, transforming growth factor-beta-1; PAI-1, endothelial-type plasminogen activator inhibitor.
Several polypeptide growth factors and cytokines that regulate cell proliferation and activity have also been shown to affect the synthesis of procollagenase. These include interleukin-1 (12, 13), platelet-derived growth factor (14, 15), epidermal growth factor (EGF) (15, 16), and tumor necrosis factor (17).

Transforming growth factor-β (TGF-β), a 25-kDa homodimeric protein with autocrine and paracrine activities, influences cellular proliferation, differentiation, and activity and is thought to be an important mediator in embryogenesis (18), wound healing, and bone remodeling (reviewed by Sporn et al. (19), Massagué (20)). Virtually all cell types studied have TGF-β cell surface receptors (21) and express TGF-β mRNA (22). TGF-β is also expressed in high abundance in mouse embryos at sites undergoing remodeling (18). Its biological activity on connective tissue cells in vitro includes the stimulation of the synthesis of extracellular matrix components such as collagen (23-27), fibronectin (24, 25, 27), and SPARC/osteonectin (24). TGF-β has also been shown to cause a fibrotic tissue response in vivo (26, 28). However, a net increase in matrix accumulation may also be contingent on the reduced activity of matrix-degrading proteinases, suppressed either in their synthesis or by proteinase inhibitors. Indeed, TGF-β has been shown to suppress the constitutive and EGF-stimulated synthesis and secretion of the lysosomal enzyme “major excreted protein” (29), a secreted thiol proteinase identified as cathepsin L (30). TGF-β also increases the synthesis of endothelial-type plasminogen activator inhibitor (PAI-1) while decreasing the synthesis in normal cells of both urokinase- and tissue-type plasminogen activators (24, 31-33), which are serine proteinases that catalyze the conversion of plasminogen to plasmin, a proteinase that can degrade many of the extracellular matrix proteins (34).

Results from the previous studies on the effects of TGF-β on the expression of collagenase and TIMP appear equivocal. Chua et al. (15) reported that TGF-β increased the synthesis of M, 60,000 and 55,000 proteins, believed to be procollagenase, in neonatal fibroblasts. However, Edwards et al. (16) have reported that TGF-β has no direct effect on the expression of fibroblast procollagenase, although TGF-β attenuated the synthesis of procollagenase mRNA levels induced by treatment of the cells with EGF or basic fibroblast growth factor. In the same study, TGF-β did not directly affect TIMP synthesis, but a synergistic increase in TIMP mRNA transcripts and TIMP synthesis was observed when TGF-β was added in combination with EGF or basic fibroblast growth factor.

The analysis of gene transcription and mRNA levels is important in the study of the regulation of protein synthesis. However, since mRNA processing, transport, and degradation before translation can affect the level of protein synthesis, the levels of cellular mRNA do not necessarily correlate with the synthesis and secretion of the protein. The cellular expression of secreted matrix metalloproteinases can be assessed by functional assays. However, since active matrix metalloproteinases irreversibly complex with TIMP (10, 11), information obtained by functional assays of medium samples containing both the inhibitor and the proteinases is of limited value since such assays measure only the net enzyme activity remaining after the TIMP is complexed with activated proteinases. Consequently, to determine actual changes in levels of the secreted proteinases by functional assay, the inhibitor must first be removed.

To investigate the effects of TGF-β on the synthesis of human fibroblast matrix metalloproteinases and TIMP, we have developed a simple, rapid two-step procedure to separate proenzymes from inhibitors present in small volumes of conditioned cell culture medium. In this paper, we report experiments that establish a regulatory role for TGF-β in the expression of fibroblast collagenolytic activity. Our findings demonstrate that TGF-β suppresses collagenase activity directly, by decreasing procollagenase synthesis, and indirectly, by increasing TIMP and PAI-1 synthesis. We also show that, in contrast to its effect on procollagenase, TGF-β induces the synthesis of 72-kDa progelatinase, characterized as a member of the matrix metalloendoproteinase family of enzymes.

**EXPERIMENTAL PROCEDURES**

**Growth Factors, Antibodies, and Reagents—** Purified human platelet TGF-β1 (35) was a kind gift from Drs. M. B. Sporn and A. B. Roberts (National Cancer Institute, National Institutes of Health). Stock solutions of TGF-β (0.2 μg/ml) were prepared in 4 mM HCl with BSA (1 mg/ml) added as carrier. cDNA to mouse TIMP (36) was generously provided by Dr. D. T. Denhardt (University of Western Ontario, London, Ontario, Canada). Rabbit anti-human TIMP antiserum was kindly provided by Dr. H. G. Weigus (The Jewish Hospital of St. Louis, MO) and rabbit anti-bovine endothelial-type PAI-1 antiserum, kindly prepared by Dr. J. Lukutowski (Scripps Institute and Research Foundation, La Jolla, CA), was a gift from Dr. S. Wasi (National Reference Laboratories, Canadian Red Cross, Ottawa, Canada).

Materials and reagents used were obtained as follows. Tissue culture supplies were from Flow Laboratories (Mississauga, Ontario, Canada), (1-14C)Glycine (ICN Radiochemicals), N-ethylmaleimide (Sigma), and dimethyl sulfoxide. 2,5-diphenyloxazole were from Du Pont-New England Nuclear. L-(35)Methionine (specific activity = 1,100 Ci/mmole) was from ICN Radiochemicals (Irvine, CA) and was stored at -80 °C. Bacterial collagenase (CLSPA) (EC 3.4.24.3) was from Worthington and further purified (37). Reagents for electrophoresis were all of electrophoresis purity and were obtained from Bio-Rad as were prestained molecular weight protein standards. Brij 35, N-ethylmaleimide (NEM), p-aminophenylmercuric acetate (APMA), and phenylmethylsulfonyl fluoride were from Sigma. Bovine serum albumin (BSA) (Pentex bovine albumin) was obtained from Laboratories (St. Louis, MO). Gelatin-Sepharose CL-4B, and protein A-Sepharose CL-4B were from Pharmacia LKB Biotechnology Inc. Pansorbin (Staphylococcus aureus) was from Behring Diagnostics (La Jolla, CA) and Nonidet P-40 was from Buthesda Research Laboratories. All other chemicals were commercially available analytical grade reagents. All solutions were prepared with distilled water further purified through a Millipore UF System (Millipore Corporation, Bedford, MA).

**Cell Culture—** Early passage human gingival fibroblasts (Gin-1; American Type Culture Collection, Rockville, MD) were plated at 8-10 × 10⁶ cells/cm² in 100-mm tissue culture dishes and grown to confluence (5-6 × 10⁵ cells/cm²) in α-minimum essential medium, pH 7.6, supplemented with 15% (v/v) heat-treated (56 °C, 20 min) fetal bovine serum and antibiotics (100 μg/ml penicillin-G, 50 μg/ml gentamicin sulfate, 0.3 μg/ml amphotericin-B) (supplemented medium) at 37 °C in a humidified atmosphere containing 10% CO2 (19, 20). Confluent cells were subcultured (1:3) every 3-4 days after treatment with trypsin (0.1 mg/ml in citrate saline) and the released cells resuspended in supplemented medium and replated as required. Experiments reported were done on cells between subcultures 6 and 9. Cell cultures were mycoplasma negative.

**Incubation with TGF-β and Isotopic Labeling—** Confluent cultures were washed twice in phosphate-buffered saline (15 mM P, pH 7.2, 0.14 M NaCl), once in α-minimum essential medium and incubated for 24 h in serum-deficient medium (α-minimum essential medium/0.2% (v/v) fetal bovine serum/antibiotics). The quiescent cultures were then incubated in the presence of 40 pM TGF-β (1.0 ng/ml), or vehicle (1 mg/ml BSA, 4 mM HCl), in 0.6 ml of serum-deficient medium and continuously labeled with 10 μCi of [35S]methionine/ml where indicated. After 24 h the medium was harvested and replaced by fresh medium with TGF-β, or vehicle, and [35S]methionine as appropriate. Medium collections were continued daily for 4 days when cell numbers were determined by Cell Counting. Experiments were performed in quadruplicate and repeated three times.

Dosimetric analyses of cell responses were performed using TGF-β at concentrations between 0.01 ng/ml and 10.0 ng/ml, or carrier, added to quiescent confluent cultures in serum-deficient medium. The dose of treatments (determined by radioactivity) of [35S]methionine/ml for 30 min and then chased for 4 h. Aliquots (10 μl) of the chase medium were analyzed by SDS-PAGE using 10% (w/v)
cross-linked polyacrylamide minishab gels as described below.

Procollagenase, 72-kDa Progelatinase, TIMP, and PAI-1 Fractionation—Procollagenase and 72-kDa progelatinase were separated from TIMP and PAI-1 by fractionating aliquots of each collection of conditioned media on tandem miniaffinity columns of heparin- and gelatin-Sepharose under conditions optimized to eliminate TIMP from the proteinase-containing eluates. After media collection, the samples were stored at 4 °C until processed (within 5 days) to avoid activation of latent enzymes by freeze-thawing. One ml aliquots of the conditioned culture media were dialyzed against chromatography buffer containing 50 mM Tris-HCl, 0.15 M NaCl, 0.5 mM CaCl2, 0.5 μg/ml Brij 35, 0.2 μg/ml NaN3, pH 7.2, at 4 °C according to Overall (38). The dialyzed samples were applied to minicolumns of 100 μl of heparin-Sepharose in 20 ml of chromatography buffer containing 1.0 M NaCl (1H fraction). Material bound to the gelatin-Sepharose, including a small amount of progelatinase, was eluted with 250 μl of chromatography buffer containing 1.0 M NaCl (G1 fraction). Following a 1 ml wash with the same buffer, 72 kDa-progelatinase and fibronectin were eluted from the gelatin-Sepharose with 490 μl of 4 X concentrated Laemmli sample buffer (39) (80 mg/ml SDS, 8 M urea, 0.5 M Tris-HCl, pH 6.8 (G2 fraction). The heparin-Sepharose minicolumns were reequilibrated with 2.0 M NaCl after 2.0 M NaCl eluted bound material from the gelatin-Sepharose buffer. However, to avoid the possibility of gelatin degradation by bound gelatinase on the gelatin-Sepharose which influence column performance in subsequent fractionations, the gelatin-Sepharose minicolumns were discarded after each use.

Collagenase, Gelatinase, and TIMP Analyses—Collagenase activity in the fractionated conditioned culture media was determined from the degradation of metabolically labeled [35]C]glycine-labeled soluble collagen (specific activity 3.5 × 106 dpm/mg) in a highly sensitive collagenase assay (3). In brief, ~6 ng of [35]C]glycine-labeled collagen (~2000 dpm/mg) was incubated with the assay buffer (50 mM Tris-HCl, 0.2 M NaCl, 5 mM CaCl2, 0.5 μl/ml Brij 35, 0.2 μg/ml NaN3, pH 7.2) in a final volume of 60 μl at 27 °C. Progelatinase was assayed after activation of the sample with 1 μM APMA for 45 min at 22 °C prior to assay. In this assay we define 1 unit of collagenase activity as that amount of enzyme which degrades 1 pg of soluble type I collagen/f at 27 °C.

The concentration of TIMP was assayed by the inhibition of a standard amount of active porcine collagenase as follows. After preincubation of 3 or 5 μl of sample with ~250 units of collagenase for 30 min at 22 °C, radiolabeled substrate was added (~2100 dpm) and the assay was incubated at 27 °C for 45 min at 22 °C before assay. Gelatinolytic activity was quantitated following the collagen reaction products by SDS-PAGE, as described below.

Gelatinase activity in conditioned medium and processed samples was quantitated by enzymeology as described below and, in unlabeled medium, by degradation of [35]C]glycine-labeled gelatin (3). Active gelatinase was assayed by incubation of 20 μl of sample at 37 °C for 2 h in assay buffer with ~5500 dpm of [35]C]glycine-labeled gelatin, prepared by heating [35]C]glycine-labeled collagen at 60 °C for 20 min. Progelatinase was activated by 1 μM APMA for 45 min at 22 °C before assay. Gelatinolytic activity was quantitated by liquid scintillation spectroscopy of the 10% (v/v) ClAcOH (1% v/v) tannic acid supernatant incubated for 18 h at 4 °C with 10 μl of either rabbit anti-human TIMP or rabbit anti-bovine PAI-1 antibody which had been coupled to 100 μl of protein-A-Sepharose. Immune complexes were collected by centrifugation (12,000 x g, 5 min) and the precipitate washed five times with immunoprecipitation buffer containing 1 mg/ml BSA. The sample containing in the precipitate was reextracted with Laemmli electrophoresis sample buffer (2 × concentrated), with or without 65 mM DTT, at 56 °C for 20 min, and an aliquot of the immunoprecipitate analyzed by fluorography after SDS-PAGE on 10% or 15% (v/v) cross-linked polyacrylamide minishab gels as described below.

Gelatin-Substrate Enzymography—Gelatinolytic proteinases were assayed by gelatin-substrate enzymography using modifications of the method of Heussen and Dowdle (42) to achieve higher sensitivity and short assay times (43). Type I collagen, heated at 56 °C for 20 min, was incorporated in 10% (v/v) cross-linked polyacrylamide minishab gels to a final concentration of 40 μg/ml. Samples (3 μl) were prepared for electrophoresis without heatin or reduction. Following electrophoresis, SDS was removed from the gels by 2.5% (v/v) Triton X-100 washes (2 × 10 min) and the gels incubated in assay buffer at 37 °C for 1 or 3 h. The reaction was stopped by a 5 min wash with 5% (v/v) acetic acid at room temperature. One well was stained with 0.1% Coomassie brilliant blue G-250, 0.2 M H3PO4, 50 mg/ml amnomium sulfate, pH 2.5, then equilibrated in 25 g/100 ml ammonium sulfate, 5% (v/v) acetic acid to fix the dye and to intensify staining. Gelatinolytic activity was detected as cleared bands against the blue-stained gelatin background. Organonemeric activation of progelatinase was achieved by incubation of samples with 1 mM APMA for 30 min at 22 °C prior to addition of electrophoretic sample buffer and electrophoresis. In each of the three time course experiments, enzymography was performed on both the unfraccionated medium and the gelatin-Sepharose bound material (G2 fraction) at various sample dilutions and incubation times to ensure that gelatin degradation by the samples was in the linear range of the assay. Quantitation was then achieved by laser densitometry at 633 nm.

The gelatinolytic activity was determined by incubation of proteinase inhibitors in the assay buffer and comparing gelatinolyis with solvent controls. The proteinase inhibitors used were: EDTA (10 mM), DTT (5 mM), phenylmethylsulfonyl fluoride (1 mM), NEM (5 mM), and TIMP (~20000 units/ml), partially purified from rat osteosarcoma (ROS 17/2.8) cultures (3). Proteolytic activity was also assayed at pH 5.5 (50 mM NaAc) and caseinase (40 μg/ml NaAc) and BSA (200 μg/ml NaAc) substrates in duplicate. Products were detected by fluorography with [35]S]methionine-labeled medium protein standards, prepared by reductive methylation of Sigma M, standards with [14C]formaldehyde (41), as follows: myosin (200 KDa), β-galactosidase (116.5 KDa), phosphorylase B (92.5 KDa), BSA (66.6 KDa), ovalbumin (43 KDa), carbonic anhydrase (30 KDa), soybean trypsin inhibitor (20.1 KDa), and α-lactalbumin (14.4 KDa).

Secreted Medium Protein, Collagen, and Fibronectin Analyses—Aliquots (10 μl) of the radiolabeled secreted proteins were analyzed by SDS-PAGE using 10% (v/v) cross-linked polyacrylamide minishab gels, with or without sample reduction (65 mM DTT), as described above. Gelatinolytic proteinases were assayed from 10% (v/v) cross-linked polyacrylamide minishab gels by liquid scintillation spectroscopy of [35]S]methionine-labeled medium protein standards after exhaustive dialysis (38) of equal volumes of medium and by precipitation of medium proteins with 10% (v/v) ClAcOH, 1%
(w/v) tannic acid at 4 °C with 1 mg of BSA added as carrier. Collagen
synthesis was determined after bacterial collagenase digestions of
conditioned medium as routinely performed in our laboratories (37).
[35S]Methionine-labeled fibronectin, quantitatively bound to gelatin-
Sepharose (24), was eluted with 4 × concentrated Laemmli sample
buffer (G2 fraction) and the radiolabeled protein quantitated by laser
densitometry after analysis by SDS-PAGE under reducing conditions
and fluorography.

RNA Isolation and Analysis—Total cellular RNA was prepared
according to Auffray and Rougeon (44), modified as described. Con-
fluent fibroblast cultures in 100-mm culture dishes were washed once
in LiCl-urea lysis buffer (30 mM sodium citrate, 0.3 M NaC1, pH 7.0), 1
mg/ml SDS and then twice (20 min) in 0.2 × SSC, 1 mg/ml SDS, at 42 °C.
RNA was then treated with 0.1 M NaAc, 5 mg/ml SDS by vortexing before extraction with
equal volumes of phenol (equilibrated with 0.3 M NaAc and 2.5 volumes of ethanol). The precipi-
tated RNA was dissolved in 20 μl of RNase-free water and 2.5
μl of the DNA sheared by brief sonication.

For RNA electrophoresis, total RNA was fractionated as described under
"Experimental Procedures." The positions of marker protein stand-
ards (M), and the expected positions of proteins of interest are
indicated. The fluorograph shows that TGF-β induced an increase in
secreted protein levels, but in particular of fibronectin, procollagens,
72-kDa proteins, PAI-1, and unidentified 41, 35, and 32-kDa proteins.
In contrast, the levels of 90-kDa proteins and a 54-kDa protein,
migrating in the position of procollagenase, were suppressed by TGF-
β.

RESULTS

Dosimetric analysis of specific proteins synthesized in the
pulse-chase experiments showed that the maximal response in
secreted protein synthesis by quiescent early passage hu-
man fibroblasts in confluent monolayer culture occurred at
1.0 ng/ml (40 pm) TGF-β, a dose expected to saturate the
high affinity TGF-β cell surface receptors. Half-maximal
stimulation occurred at 0.01 ng/ml TGF-β but at 10.0 ng/ml
t of TGF-β, the stimulation in protein synthesis was slightly less
(−5%) than at 1.0 ng/ml TGF-β. Therefore, experiments were
performed using 1.0 ng/ml TGF-β, replenished daily for 4
days. Anchorage-dependent growth of the fibroblasts was
unaffected by the addition of 1.0 ng/ml TGF-β over 4 days in
culture as found in previous studies using a single addition of
1.0 ng/ml TGF-β (23).

TGF-β increased the levels of [35S]methionine-labeled se-
creted proteins 1.4–1.6-fold in a time-dependent manner, an
effect that is not due to an increased cellular uptake of the
radiolabeled amino acid (23). Electrophoretic separation and
fluorographic analysis of the secreted proteins in time course
experiments (Fig. 1) revealed that the levels of certain pro-
tains were selectively stimulated by TGF-β. Several of these
proteins were identified after further analysis as discussed below.
Notably, over 4 days, PAI-1 levels were increased >10-fold
at all time points from essentially undetectable levels in
control cell media, fibronectin was increased from 2.3–2.7-
fold throughout the 4 days, and procollagens showed a 1.4-
and 1.9-fold increase at days 2 and 4, respectively, similar to
previous reports using single additions of TGF-β (23–27).
The levels of 72-kDa proteins were also increased by TGF-β, one
of which was identified as progelatinase after being resolved
from another 72-kDa protein when electrophoresed under
nonreduced conditions (see below). Based upon the M, of the
reduced form of the progelatinase and the characterization
described below, we refer to the enzyme as 72-kDa progelatin-
ase. The levels of unidentified 41-, 35-, and 32-kDa proteins
were also increased by TGF-β treatment over the 4-day ex-
periments. In contrast, proteins which electrophoresed in the
90-kDa region and a 54-kDa protein were reduced in amount in
TGF-β-treated cultures over the time course.

The effect of TGF-β on the synthesis of collagenolytic
proteinases was investigated using functional assays and met-
allic labeling. No active collagenase and only small amounts
of procollagenase were detected in unfractionated conditioned
medium despite the use of a highly sensitive functional assay
which utilized metabolically labeled collagen of high specific
activity. However, the conditioned medium was also shown to
contain TIMP which will specifically inhibit collagenase and
related metalloendoproteinases. To assay enzymes effectively,
procollagenase and the 72-kDa progelatinase were first sepa-
rated from the TIMP by fractionating aliquots of each collec-
tion of conditioned media on tandem miniaffinity columns
under conditions optimized to eliminate TIMP from the proteinase-containing eluants. The NaCl concentration of the
chromatography buffer was found to be crucial for this frac-
tionation and varied with the source of the enzyme. For these
studies, when the salt concentration was increased to 0.25 M
NaCl, TIMP did not bind to either of the affinity columns and
was quantitatively recovered with PAI-1 in the unbound
fraction. Under these conditions, it was consistently found that ~80% of the total recoverable procollagenase in the
conditioned medium samples was bound to and could be eluted

![Fig. 1. Effects of TGF-β on secreted [35S]methionine-la-
beled proteins secreted by human fibroblasts. Aliquots (10 μl)
of [35S]methionine-labeled conditioned culture medium from human
fibroblasts maintained for 4 days (1–4) in the absence (C) or presence
(T) of 1.0 ng/ml TGF-β were analyzed by SDS-PAGE on 10% (w/v)
cross-linked polyacrylamide minislab gels under both reduced
(−DTT) and nonreduced (+DTT) conditions as described un-
der "Experimental Procedures." The positions of marker protein stand-
ards (M), and the expected positions of proteins of interest are
indicated. The fluorograph shows that TGF-β induced an increase in
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In contrast, the levels of 90-kDa proteins and a 54-kDa protein,
migrating in the position of procollagenase, were suppressed by TGF-
β.](image-url)
from the heparin-Sepharose with 1.0 M NaCl in the H1 fraction. The remaining 20% of the procollagenase was recovered from the gelatin-Sepharose with 1.0 M NaCl (G1 fraction). Increasing the salt concentration above 0.25 M resulted in a progressive decrease in the binding of procollagenase to the heparin-Sepharose and little recovery of the enzyme. In contrast to the procollagenase, only a small percentage (7%) of the total 72-kDa progelatinase bound to heparin-Sepharose in 0.25 M NaCl. However the 72-kDa progelatinase, as well as fibronectin, showed high affinity for gelatin-Sepharose in 0.25 M NaCl with only ~3% eluting in 1.0 M NaCl (G1 fraction) and 90% being recovered in the G2 fraction.

Eluants from each column (H1 and G1) were assayed both separately and pooled to give total procollagenase activity for each sample (Fig. 2). In control assays, collagen substrate was not degraded following incubation in the presence of TGF-β, fresh medium, or trypsin (enzyme/substrate ratios of 10:1). Further, active collagenase was not detected in any of the processed medium fractions. In three separate experiments TGF-β suppressed procollagenase levels progressively over 4 days as shown for one experiment in Fig. 3. A reduction in procollagenase levels of ~50%, compared with controls, was observed on day 1. On day 2 procollagenase was further reduced (~75% reduction) and was undetectable on days 3 and 4. Moreover, the TGF-β suppression of procollagenase levels occurred despite a general 1.4–1.6-fold increase in secreted protein synthesis as determined by radioactivity in fresh medium, or trypsin (enzyme/substrate ratios of 10:1).

Eluants from each column (H1 and G1) were assayed both

Increasing the salt concentration above 0.25 M resulted in a progressive decrease in the binding of procollagenase to the heparin-Sepharose eluents was activated by 1 mM APMA and incubated with ~2100 dpm [14C]glycine-labeled I collagen; S, substrate control. Active collagenase was assayed in the absence of APMA (−APMA), assays of which are shown for TGF-β treated cells at each time point. The collagen reaction products were resolved by SDS-PAGE on 7.5% (w/v) cross-linked polyacrylamide minigel plates. The fluorograph shows that fibroblast procollagenase activity, as evidenced by the generation of collagenase-specific 72-kDa (αA-chain) fragments, was suppressed by TGF-β over 4 days.

**Fig. 2.** Collagenase expression in fibroblast cultures stimulated with TGF-β. Human fibroblasts were maintained in serum-deficient medium in the absence (C) or presence (T) of 1.0 ng/ml TGF-β for 4 days (1–4). At the times indicated, culture media were harvested and fractionated prior to assaying for procollagenase as described under “Experimental Procedures.” Procollagenase in the heparin-Sepharose eluents was activated by 1 mM APMA and incubated with ~2100 dpm [14C]glycine-labeled type I collagen; S, substrate control. Active collagenase was assayed in the absence of APMA (−APMA), assays of which are shown for TGF-β treated cells at each time point. The collagen reaction products were resolved by SDS-PAGE on 7.5% (w/v) cross-linked polyacrylamide minigels.

The fluorograph shows that fibroblast procollagenase activity, as evidenced by the generation of collagenase-specific 72-kDa (αA-chain) fragments, was suppressed by TGF-β over 4 days.

The fluorograph shows that fibroblast procollagenase activity, as evidenced by the generation of collagenase-specific 72-kDa protein, was markedly increased by TGF-β when analyzed under reduced conditions in both the continuous labeling (Fig. 1, +DTT) and pulse-chase experiments. Under nonreduced electrophoretic conditions the 72-kDa band was resolved into two proteins which electrophoresed with M, values of 72,000 and 66,000 (calculated using M, values of reduced marker proteins). When analyzed in this way it was found that almost the entire TGF-β-induced increase in the level of the 72-kDa band in reduced gels could be accounted for by a 6.5–7.5-fold increase in the levels of an unidentified protein designated here as 72-kDa protein. In contrast, the 66,000 protein, which was identified as the gelatinase when analyzed by enzymography (Fig. 5), was increased 1.6–1.8-fold by TGF-β treatment. That the unidentified 72-kDa protein appears unrelated to the 72-kDa progelatinase was indicated by a lack of gelatinolytic activity (Fig. 4) and, unlike the gelatinase, the 72-kDa protein did not bind to gelatin-Sepharose (Fig. 5) and was recovered in the unbound fraction (not shown). The increased synthesis of both the 72-kDa progelatinase and the 72-kDa protein could be stimulated by brief exposure (1 h) to 1.0 ng/ml TGF-β both in the presence or absence of 100 μg/ml cycloheximide (a dose that inhibited 95% of protein synthesis in these cells). This indicated that the increased synthesis of both the 72-kDa progelatinase and 72-kDa protein by TGF-β did not require de novo synthesis of regulatory protein.

Since a number of proteolytic enzymes have gelatinolytic activity it was important to characterize the enzyme analyzed in these studies to determine the relevance of the TGF-β
throughout the entire thickness of the 10% cross-linked polyacrylamide minislab gels under nonreduced conditions, and assayed for gelatinase activity by enzymography (1 h incubation) as described under “Experimental Procedures.” Gels were stained with 0.1% (w/v) Coomassie Brilliant Blue G-250 in 0.2 M H₃PO₄, 50 mg/ml ammonium sulfate. In the top panel, the p-gelatinase band can be identified in the fluorograph of medium proteins from day 3 control and TGF-β-treated cells also electrophoresed on gelatin (40 μg/ml) substrate, 10% (w/v) cross-linked polyacrylamide minislab gels under nonreduced conditions. Samples were also assayed for gelatinase activity after 1 mM APMA activation (30 min) of latent enzymes before electrophoresis (+APMA). APMA activation generated a 62-kDa form of gelatinase, labeled p-gelatinase. A minor 59-kDa gelatinase band (gelatinase) was also resolved. The fluorograph in the lower panel is of medium protein from control cells that was treated with (+) or without (−) APMA showing the generation of a 62-kDa protein in APMA-treated samples. The positions of reduced Mᵣ marker proteins are indicated. BSA, (A) added as carrier for TGF-β and in vehicle controls is labeled in the nonreduced samples.

effects on connective tissue breakdown. The 72-kDa progelatinase was characterized as a neutral metalloendoproteinase as shown by the use of proteinase inhibitors incorporated in the assay buffer (Fig. 6). Both EDTA and DTT totally inhibited gelatinolytic activity whereas phenylmethylsulfonyl fluoride and NEM were not inhibitory; indeed, enhanced proteolytic activity and revealed the presence of a minor 49-kDa gelatinolytic activity. Importantly, the proteinases were also inhibited by partially purified rat TIMP although complete inhibition was not achieved. Since it would take some time for TIMP, a 31-kDa protein, to diffuse with the conditioned medium, harvested daily for bound avidly to gelatin-Sepharose, requiring the presence of gelatin-binding proteins were eluted with 80 mg/ml SDS, 8 M urea, 0.5 M Tris-HCl, pH 6.8 (G2 fraction), and electrophoresed without reduction on 10% (w/v) polyacrylamide minislab gels containing 40 μg/ml gelatin (upper panel). The eluant contained two proteins, fibronectin and a 66-kDa protein which was identified by enzymography (E) as the gelatinase (p-gelatinase). Under reduced conditions the 66-kDa protein electrophoresed with an apparent Mᵣ of 72,000 and lost gelatinolytic activity. Mᵣ, reduced Mᵣ, protein markers. Lower panel, the affinity purified 72-kDa progelatinase was quantitated by laser densitometry at 633 nm (n = 4). Data expressed as the mean ± S.D. (n = 4). As analyzed by the two-tailed Students t test, the differences in levels of affinity purified 72-kDa progelatinase between control and TGF-β treatments were significant for all days with p < 0.05 for day 1 and p < 0.005 for days 2, 3, and 4.

**Fig. 5. Temporal effects of TGF-β on the biosynthesis of gelatin-binding proteins in human fibroblasts in culture.**[35S] Methionine-labeled conditioned medium, collected over 4 days (1–4) from cultures incubated in the continuous presence of 1.0 ng/ml TGF-β (T), or carrier (C) in serum-deficient conditions, were chromatographed on tandem heparin-Sepharose and gelatin-Sepharose minicolumns as described under “Experimental Procedures.” The gelatin-binding proteins were eluted with 80 mg/ml SDS, 8 M urea, 0.5 M Tris-HCl, pH 6.8 (G2 fraction), and electrophoresed without reduction on 10% (w/v) polyacrylamide minislab gels containing 40 μg/ml gelatin (upper panel). The eluant contained two proteins, fibronectin and a 66-kDa protein which was identified by enzymography (E) as the gelatinase (p-gelatinase). Under reduced conditions the 66-kDa protein electrophoresed with an apparent Mᵣ of 72,000 and lost gelatinolytic activity. Mᵣ, reduced Mᵣ, protein markers. Lower panel, the affinity purified 72-kDa progelatinase was quantitated by laser densitometry at 633 nm (n = 4). Data expressed as the mean ± S.D. (n = 4). As analyzed by the two-tailed Students t test, the differences in levels of affinity purified 72-kDa progelatinase between control and TGF-β treatments were significant for all days with p < 0.05 for day 1 and p < 0.005 for days 2, 3, and 4.

**Fig. 6. Identification of the fibroblast 72-kDa progelatinase as a neutral metalloendoproteinase.** Aliquots (1.5 μl) of conditioned medium collected from confluent cultures of human fibroblasts were treated with (+) or without (−) 1 mM APMA prior to electrophoresis on 40 μg/ml gelatin, 10% (w/v) cross-linked polyacrylamide minislab gels and analyzed by enzymography (3 h incubation) as described under “Experimental Procedures.” Prestained reduced marker proteins (Mᵣ) and the position of albumin (A), nonreduced, added to culture medium as carrier, are shown. Enzymography was performed in assay buffer alone (control) or in the presence of the following reagents: 10 mM EDTA, 5 mM DTT, 1 mM PMSF, 5 mM NEM, and 20,000 units of TIMP. Enzymography of 10-μl aliquots of conditioned medium was also performed using 40 μg/ml casein-substrate gels.

indicated from the minimal degradation (3%) after 2 h of ~10 ng of [3H]glucose-labeled gelatin by 20-μl samples in gelatinase assays. However, after enzyme activation by the organomercurial APMA, 66% degradation of the [3H]glucine-labeled gelatin occurred. Thus, it is likely that the 66-kDa form is a zymogen. Activation of enzymes in the conditioned me-
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dium samples with APMA caused a 4 kDa decrease in $M_r$ of some of the 66-kDa p-gelatinase to a 62-kDa form we termed p'-gelatinase (Figs. 4 and 6). However, the sensitivity of the 66-kDa form to APMA was only observed when the enzyme was in the native form, as found in the conditioned medium or in the G1 fraction, but not when eluted and treated under denaturing conditions (G2 fraction). APMA treatment also resulted in the generation of a new band in fluorographs anodal to the 72-kDa progelatinase band that is likely to represent the p'-gelatinase (Fig. 4). When prolonged incubation times were used (6 h) a 59-kDa form was detected as a minor component in unactivated samples, possibly representing a trace amount of the naturally activated form of the enzyme, which we have designated in the figures as gelatinase. Gelatinolytic activity was sometimes evident in the 134 kDa region (Fig. 6), possibly representing dimers of the enzyme, which disappeared following APMA treatment prior to electrophoresis. Thus the 72-kDa progelatinase is likely to be a gelatinase in the matrix metalloendoproteinase family of enzymes.

The effect of TGF-β on the expression of proteinase inhibitors was also investigated. In the continual presence of TGF-β, human fibroblasts synthesized increased amounts of TIMP and PAI-1 as determined by several criteria. Specific immunoprecipitation of [35S]methionine-labeled secreted proteins showed that TGF-β increased the levels of TIMP, a 31-kDa protein, 1.6-fold (±0.3, mean ± S.D.) and PAI-1, a 48-kDa protein, >10-fold over the 4-day experiment (Fig. 7). Whereas the marked increase in the level of PAI-1 was evident at day 1, peaking at day 2 and was maintained at high levels over controls throughout the time course, the maximal induction of TIMP appeared delayed, occurring at day 2. Similar results were also obtained when TIMP expression was quantitated in conditioned culture medium by functional assays (Fig. 8). We also examined the changes in TIMP mRNA levels in response to TGF-β (Fig. 9). TIMP mRNA, identified in the total cellular mRNA isolated at days 1 and 4 after stimulation of quiescent, confluent human fibroblasts with 1.0 ng/ml TGF-β, was only slightly stimulated at day 1 (1.3–1.4-fold) compared with a 2.4-fold increase at day 4.

**DISCUSSION**

To study the effects of TGF-β on the expression of collagenase in human fibroblasts, we first separated the enzyme from TIMP in the conditioned medium using tandem miniaffinity columns. This procedure allowed the subsequent quantitation of the enzyme using a functional assay. Using this assay, the synthesis of procollagenase was found to be progressively decreased to undetectable levels 3 and 4 days after the daily addition of TGF-β. This suppression occurred despite a 1.4–1.6-fold increase in the synthesis of secreted protein, demonstrating more definitively the selectivity of the effects of TGF-β on protein synthesis by fibroblasts reported previously (24). Our findings differ from those of Edwards et al. (16) who found that TGF-β had no effect on medium procollagenase levels nor on the steady-state levels of procollagen mRNA in late passage (subculture 24–34) fetal lung fibroblasts. However, in the functional assays reported by Edwards et al. (16) the effect of TGF on the enzymes activated in the assays of conditioned medium was not considered. Further, in contrast to our studies and previously published work (23–28), Edwards et al. (16) found no effect of TGF-β on the synthesis of matrix proteins. In our studies, using early passage (subculture 6–9) human gingival fibroblasts, we found decreased levels of both procollagens (1.9-fold) and fibronectin (~2.5-fold) in the conditioned media. Nonetheless, Edwards et al. (16) did report that TGF-β attenuated the basic fibroblast growth factor and EGF-induced increases in mRNA for procollagenase. Our data also contrast the interpretation of an earlier study in which TGF-β was reported to induce the synthesis in neonatal skin fibroblasts of 60- and 55-kDa proteins believed, but not directly demonstrated, to be procollagenase (15).

A characteristic action of TGF-β on mesenchymal cells is to elevate the synthesis and deposition of connective tissue matrix components (23–28). However, the net accumulation of matrix is also dependent upon its rate of degradation. The decreased synthesis of procollagenase following TGF-β treatment of human fibroblasts reported here, reveals a concerted dual action of TGF-β in the regulation of connective tissue matrix accumulation. In particular, the decreased synthesis of procollagenase by TGF-β may augment the TGF-β-induced elevation in synthesis of collagen by fibroblasts in circumstances where the extracellular accumulation of procollagenase may otherwise favor the degradation of collagen. TGF-β has also been reported to antagonize both the EGF-induced increase in transin mRNA levels (46), the rat homologue of human stromelysin (47), and the synthesis of the major excreted protein identified as cathepsin L (30), by Swiss 3T3 cells and human fibroblasts (29). It is of interest that cathepsin L, which is optimally active at acidic pI, is the major collagenolytic cathepsin (48). Consequently, it appears that...
was isolated from quiescent confluent fibroblast cultures at days 1 proteinase that cleaved denatured collagen α-chains, but not mRNA as described under “Experimental Procedures.”

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the catheptic collagenolytic pathway which can operate at the proteinase collagenolytic pathway active at neutral pH and @.

TGF-α can suppress both of the major extracellular collagenolytic pathways that are believed to operate during inflammation and wound healing (1); that is, the matrix metalloendopeptidase collagenolytic pathway that occurs naturally compared to the site of cleavage that occurs by TGF-β does not require concomitant 4 k decrease in Mr. The gelatinase appears similar to the gelatinase from rabbit calvaria (4) and is probably identical to the gelatinase purified from human gingival fibroblasts (5). Moreover, based on the cleavage of type IV collagen and similar physical properties, the 72-kDa progelatinase may be related to the type IV collagen-degrading metalloendopeptidase described by Salo et al. (49).

When the 72-kDa progelatinase was electrophoresed under nonreduced conditions it migrated with an apparent Mr of 66,000, indicating the presence of intramolecular disulfide cross-link(s). On gelatin-substrate gels, three closely spaced bands of gelatinase activity were resolved under the nonreduced conditions. The major 66-kDa form (p-gelatinase in figures) is likely to be thezymogenform of the enzyme since in [14C]glycine-labeled gelatin assays essentially no activity was detected unless the enzyme was activated by APMA. That thezymogenform was active in enzymography assays is consistent with the activation of the related metalloendopeptidases, collagenase, and stromelysin, with SDS (7, 50, 51). The difference in Mr, values of the gelatinase generated by APMA treatment (the 62-kDa form, p-gelatinase) and the 59-kDa form (gelatinase) appears to be due to differences in the site of cleavage that occurs naturally compared to the in vitro activation by APMA. Similarly, different forms of activated collagenase (52), stromelysin (51), and endothelial gelatinases (51) have also been described.

Although TGF-β reduces stromelysin (transin) mRNA levels (46), it is of interest that two closely related matrix metalloendopeptidases, procollagenase and 72-kDa progelatinase, are not regulated in a coordinate manner by TGF-β, contrasting the co-ordinated control of collagenase and stromelysin by other factors (e.g. 51). The increase in 72-kDa progelatinase levels by TGF-β occurred with as little as a 1-h pulse of TGF-β and was not inhibited by cycloheximide. This indicates that the response of the 72-kDa progelatinase gene to TGF-β does not require de novo protein synthesis, as has been found for the matrix protein fibronectin.3 The elevated synthesis of 72-kDa progelatinase (~1.7-fold) points to a potentially important role for this enzyme in the formation of newly deposited connective tissue, a process that occurs rapidly during wound healing. Since the 72-kDa progelatinase is not active on intact native collagen, its increased synthesis does not necessarily contradict the connective tissue formative effects of TGF-β. Indeed, the activity of the gelatinase in removing unfolded or abnormal collagen may facilitate the

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formation of a functional connective tissue from an initial repair matrix.

We have also found that TGF-β increased TIMP expression in human fibroblasts as shown by analysis of TIMP mRNA, immunoprecipitable protein, and biological activity in the conditioned medium. The stimulation of TIMP mRNA and immunoprecipitable protein levels appeared to exceed the expression of the functional protein. Immunoprecipitable TIMP was increased ~1.6-fold by TGF-β with maximal levels occurring on day 2. TIMP message levels were also increased in TGF-β-treated cultures where a 2.4-fold increase was found after 4 days, contrasting a 1.3-1.4-fold increase in TIMP mRNA on day 1. In separate experiments, the 1.7-fold increase of functional TIMP in the medium was a delayed effect, occurring after the second day of TGF-β treatment. mRNA processing, transport, and degradation before translation can affect the level of protein synthesis, as shown for other TGF-β-regulated genes including collagen and fibronectin (27) and urokinase-type plasminogen activator (39). The delay in the maximal stimulation of TIMP mRNA, immunoprecipitable protein, and biologically active TIMP by TGF-β indicates that the regulation of TIMP mRNA and protein levels could involve a secondary response to TGF-β. Notably, Edwards et al. (16) reported that TGF-β did not significantly induce TIMP mRNA levels in late passage human lung fibroblasts after 12 h, but observed synergistic increases in the TIMP mRNA transcript with TGF-β that had been induced by EGF and basic fibroblast growth factor.

TGF-β regulation of PAI-1, a serine proteinase inhibitor, differed markedly from TIMP. PAI-1 levels were elevated over controls >10-fold at day 1, increasing further at day 2, and were then maintained at high levels at days 3 and 4 in the continuous presence of TGF-β. Previous studies have shown that PAI-1 mRNA is rapidly increased in cells following TGF-β treatment (32) and that PAI-1 synthesis is stimulated by TGF-β in fibroblasts, epithelial, and endothelial cells (31-33). Thus, the effect of TGF-β on PAI-1 expression is in agreement with previous results using other cells. We further show that the stimulation of PAI-1 synthesis is maintained following daily administration of TGF-β, and the cells do not become refractory to the continuous presence of TGF-β, contrasting the rapid increase and fall-off in PAI-1 levels in culture medium following a single addition of TGF-β (32).

The decreased expression of net plasminogen activator activity by normal cells in response to TGF-β has important consequences in regulating the activity of matrix degrading proteinases. The activation of the proenzyme plasminogen, to plasmin, by plasminogen activator would be reduced with a concomitant reduction in tissue levels of plasmin, a proteinase having a broad substrate specificity (34). Moreover, since plasmin can activate procollagenase (53, 54), TGF-β may also reduce collagenase activity indirectly. The rapid synthesis and secretion of PAI-1 by cells in response to TGF-β might have the effect of blocking the activation of any procollagenase already present in the tissue. This would be complemented by the subsequent reduced synthesis of procollagenase. The increased secretion of TIMP and PAI-1 would stabilize the new matrix induced by TGF-β. However, the delayed induction of TIMP may nonetheless allow the early degradation of any abnormal, newly synthesized, or partially unfolded collagens by the activity of the 72-kDa progelatinase, increased by TGF-β, in the new matrix. A scheme summarizing the possible modulation of fibroblast collagenolytic activity by TGF-β is shown in Fig. 10.

An overall suppression of proteolytic pathways favoring net matrix accumulation appears to be a general action of TGF-β in normal cells. In addition to the effects reported here of TGF-β on collagenase, TIMP, and PAI-1 synthesis, TGF-β has been shown to reduce net plasminogen activator activity through a decreased synthesis of urokinase- and tissue-type plasminogen activators (31-33). TGF-β also acts antagonistically to EGF, not only in blocking the EGF-induced suppression of matrix protein synthesis (23), but also to reduce the EGF-induced increase in plasminogen activator activity (31), mRNA levels for stromelysin (transin) (46), and cathepsin L (major extracellular protein) synthesis (29). The stimulation by TGF-β of the synthesis of extracellular matrix proteins by cells in culture (23-27), the promotion of wound healing (28), fibrotic, and angiogenic responses following subcutaneous injection (26), its presence in large quantities in platelets (2-3 µg/gm) (35), and the synthesis and secretion of TGF-β by activated macrophages (55) and lymphocytes (26), together indicate potentially important regulatory roles for TGF-β in inflammation and wound healing. The localization of TGF-β in many tissues of the mouse embryo and particularly those tissues undergoing extensive remodeling (18) also indicate the importance of TGF-β in development. Our findings, which have shown that TGF-β reduced the levels of procollagenase, stimulated the accumulation of TIMP in the conditioned culture medium, increased the synthesis of 72-kDa progelatinase and, in confirmation of previous work, increased PAI-1 synthesis, are consistent with the promotion of connective tissue matrix formation by TGF-β and provide evidence supporting the proposed role of TGF-β as a key mediator of connective tissue formation and remodeling in both soft and mineralized connective tissues.

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REFERENCES


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